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In Memoriam

EUGENE CURTIS AUCHTER

1889–1952

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1889–1952

Eugene Curtis Auchter was born in Elmgrove, New York, September 14, 1889. His boyhood was spent in the center of a fruit growing region in western New York where his interest in tree fruits and plant propagation had its beginning. After graduating from high school in Spencerport, New York, he entered Cornell University in 1908. His early interest in the problems of fruit production led to his choice of pomology as a major in his academic training. He was appointed student assistant in pomology in 1911 and received his B.S. degree in 1912. His Master's degree in pomology was conferred by Cornell in 1918.

In 1914 he joined the faculty of West Virginia University as instructor in horticulture. While there, his research was largely in the field of growth response of apples to pruning and soil management.

In 1918 he became Head of the Department of Horticulture at the University of Maryland. During the next 10 years he carried on a vigorous program of graduate teaching and training of young men in horticultural research. He emphasized plant physiology in his studies on mineral nutrition, pollination, and tree growth in solving some of the practical problems of the fruit growers. He returned to Cornell University for further graduate training in plant physiology, genetics, and soils, and received his Ph.D. in 1923. His Doctor's dissertation entitled "Is There Normally a Cross-Transfer of Foods, Water, and Mineral Nutrients in Woody Plants," published in 1923, was an important contribution to the physiology of fruit plants.

Many promising students came to Maryland for graduate study under Dr. Auchter's direction. Graduates from the University of Maryland trained by him now hold important research positions in state universities and federal laboratories in this country and abroad.

In 1928 he was appointed to the position of Principal Pomologist in the Bureau of Plant Industry of the U. S. Department of Agriculture. Here he was given the responsibility of reorganizing the horticultural research program of the Department. Under his leadership important research contributions were made in the physiology, cytology, and pathology of fruit, vegetable, and ornamental crops.

In 1936 he established in the Division of Fruit and Vegetable Crops important basic research studies in the fields of photoperiodism and plant hormones.

Dr. Auchter possessed marked administrative ability and made a keen analysis of research programs. His advice and counsel were sought on research planning in many fields.

He became Assistant Chief of the Bureau in 1935 and Chief in 1938. In 1942, under Executive Order setting up the Agricultural Research Administration in the Department of Agriculture, he was made its first Administrator. During his tenure as Chief of Bureau, and later as Research Administrator, the Plant Industry Research Center was established at Beltsville, Maryland.

He resigned from the Department of Agriculture in 1946 to become Director of the Pineapple Research Institute of Hawaii.

He was a member of various scientific societies: Sigma Xi; American Association for the Advancement of Science; American Society for Horticulture Science, of which he was president in 1926; the American Society of Plant Physiologists; Phytopathology and the American Genetic Association; Washington Academy of Science; honorary fellow of the Royal Horticultural Society of England; Botanical Society of Hawaii; and officer of the Pacific Science Council.

In 1951 he was the recipient of the Wilder Medal awarded by the American Pomological Society with the following citation:

> "Scientist, educator, administrator, leader in horticulture, through whose vision and devotion opportunities have been provided for substantial advancement in horticultural research of great benefit to society."

Dr. Auchter died at his home, 4471 Kahala Avenue, Honolulu, T. H., July 8, 1952. His wife Catherine Beaumont Auchter survives.

F. P. CULLINAN

Beltsville, Maryland

PLANT PHYSIOLOGY

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ENZYMATIC ACTIVITIES AS INDICATIONS OF COPPER AND IRON DEFICIENCIES IN PLANTS

J. C. Brown and S. B. Hendricks

(WITH FIVE FIGURES)

Received May 26, 1952

Attempts to determine the adequacy of various elements in the nutrition of plants before harvest have largely depended upon the appearance of deficiency symptoms or upon elemental analyses of tissues (**6, 8, 14, 16**). Deviations from normal, however, either in appearance or composition, often develop only when the deficiency is extreme. A new approach is presented here. It is based upon the hypothesis that if an element is limiting in the nutrition of a plant, the deficiency will be evident in the changed activity of an enzyme among those requiring the element for their function. Elements essential solely in enzyme action and which are not readily dissociated from the protein moiety will be considered. These include iron when present in porphyrins, copper, zinc, and manganese, but not potassium and calcium. The postulate implies a pattern among the enzymes containing iron, for example, in which as iron becomes deficient the aberrant behavior of one, rather than all, of the iron enzymes will first limit growth. Accordingly, to test the postulate, activities of a number of enzymes requiring a particular element should be measured as the supply of that element in the plant is varied from normal to deficient levels. If the hypothesis is valid, then measurement of the activity of the critical enzyme might best be used to discover a deficiency.

Nutrition with respect to copper and iron, which are required in electron transferring enzymes including terminal oxidases, will be considered. The concentration of iron and copper in plant tissue has not been a satisfactory index of deficiency (**5, 9, 10, 11, 13**). Twyman (**15**) found a wide range of iron concentration in iron-deficient plants with accumulation in excess of normal plants in some cases. Bailey and McHargue (**1**) found the copper concentration of plants showing severe copper deficiency to be somewhat greater than that of normal plants.

Methods and materials

Plants were grown in two-gallon pots in the greenhouse on soils at various levels of iron and copper supplying capacity. These levels were

obtained by mixing an organic soil low in copper supplying capacity but adequate in iron with a soil giving rise to lime-induced chlorosis. The following combinations of the two soils by volume were used: Soil 1, calcareous, pH 7.8; soil 2, one third organic, two thirds calcareous, pH 7.5; soil 3, two thirds organic, one third calcareous, pH 7.2; soil 4, organic, pH 6.0; and soil 5, organic with added $CuSO_4 \cdot 5\ H_2O$.

Soils 1, 2, and 3 received a 10–38–8 fertilizer at the rate of 750 p.p.m. and soils 4 and 5 received 6,000 p.p.m. of a 3–9–18 fertilizer. Micronutrient elements were added to soils 4 and 5 in the form of ferrous sulphate and manganous acetate at 100 p.p.m., and zinc acetate, boric acid, and cobaltous chloride at 50 p.p.m. Copper sulphate at the rate of 400 p.p.m. was also added to soil 5 in order that copper would not be limiting in nutrition of plants grown on that soil. After thorough intermixing of the fertilizer, the soils were brought to equal moisture content and allowed to stand for one week before planting.

Experiments were also carried out on a freshly limed, acid soil, a Chester silt loam to which increasing amounts of precipitated chalk were added as follows: Soil A, none, pH 5.1; soil B, 2500 p.p.m., pH 6.6; soil C, 7500 p.p.m., pH 7.2; soil D, 15,000 p.p.m., pH 7.3; soil E, 30,000 p.p.m., pH 7.4. An 8–12–14 fertilizer was mixed with each of these at the rate of 600 p.p.m. The soils were then brought to equal moisture content and held in the pots for three weeks before planting.

The following plants were selected for their varying response to iron and copper nūtrītion: Lupines, white (albus), blue (angustifolius) and yellow (luteus); soybeans, Hawkeye and PI-54619-5-1; corn, Minnesota Hybrid no. 800; wheat, Thatcher; and tobacco, Connecticut broadleaf. Lupines and soybeans were grown on all the soils while corn, wheat, and tobacco were used only on soils 1 to 5.

Assays for ascorbic acid oxidase, catalase, and peroxidase were carried out under fixed procedures for measurement and extraction from the plant. Leaves were taken from the upper part of the plants at several stages of growth without attention to the light conditions at the time of harvest. They were cut with scissors and composited after which catalase activity was measured. The gasometric procedure described by Bailey and McHargue (**2**) was followed except that the tissue was macerated in a blender for two and one half minutes with ice present. Peroxidase activities were determined colorimetrically and activities of ascorbic acid oxidase by the manometric method essentially as outlined by James and Cragg (**7**), except that the tissue sample in the case of ascorbic acid oxidase was reduced to 10 gm., ascorbic acid concentrations were increased to 4 mg./ml., and air was used in the manometric flasks. The leaves for these determinations were frozen immediately after cutting and held frozen overnight, as required by the schedule of work and demonstrated to be a safe procedure. Oxidation of ascorbic acid measured on boiled tissue in a few cases was about one fourth that of the fresh tissue. Duplicate determinations were run on

a number of the samples, and the reproducibility of the results was tested with harvested replicates of wheat growing on soils 1 to 5. Since the variance was low, single determinations were made at each harvest in most instances in order that activities of the several enzymes could be measured from the same treatments at three stages of growth.

Results

Development of chlorosis in the three varieties of lupines growing on soils 1, 2, 3, and 4 varied with the variety and soil as shown in figure 1. All varieties were chlorotic on the naturally calcareous soil, but the white variety was markedly less susceptible than the yellow. The plants grew normally on the organic soil to which copper had been added (soil 5). Thatcher

FIG. 1. Chlorosis of three lupine species resulting from limited iron supply. Front row, yellow (luteus); center, blue (angustifolium); rear, white (albus). Soils 1 to 5 as indicated. Results for soil 4 were similar to those for 5.

spring wheat, on the other hand, grew normally on the naturally calcareous soil and was chlorotic, showing die back of leaf tips, on the organic soil deficient in copper (soil 4) as shown in figure 2.

Both varieties of soybeans were retarded in growth on the naturally calcareous soil, but only the PI-54619-5-1 variety was chlorotic. Susceptibility of soybean PI-54619-5-1 to chlorosis was found by Dr. M. G. Weiss to be determined by a single major gene (**18**). Connecticut broadleaf tobacco was also retarded in growth and chlorotic on the naturally calcareous soil. Blue and yellow lupines growing on the limed Chester silt loam (E) developed chlorosis. The white variety and the two soybean varieties grew normally on these limed soils (B to E).

Comparative activities of ascorbic acid oxidase, catalase, and peroxidase for the various plants under the several conditions of growth are shown in

the tables and by the bar graphs in the figures. Green weights for the several harvests on soils 1 to 5 are also listed. No significant differences in green weight were noted for soybeans or lupines growing on the limed Chester soil (A to E).

Discussion

Results on the relative activities of ascorbic acid oxidase and catalase are correlated with development of deficiency symptoms and reduction of yield of the various crops according to a pattern that might well be determined by the terminal oxidases, even though these enzymes and their distribution are still unknown in most cases. Similarly they reflect the degree to which the supply of copper or iron is limited even though this limited sup-

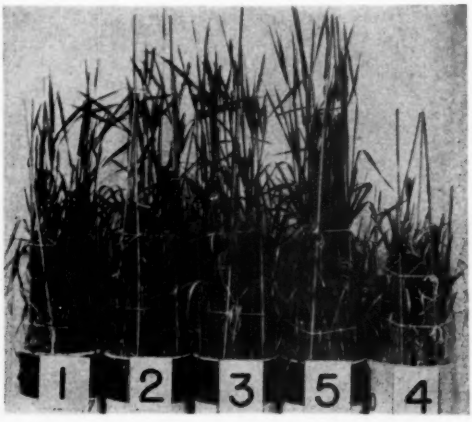

FIG. 2. Thatcher wheat on soils 1 to 5. Plants with limited copper supply on soil 4 are chlorotic and retarded in growth.

ply does not necessarily result in development of a deficiency symptom or a reduction in yield. None of the crops suffered deficiency for both copper and iron under these conditions of limited supply, but all suffered from iron or copper deficiency.

The most striking result is the very low activity of ascorbic acid oxidase in corn and wheat grown with limited copper supply on the organic soil 4 as shown by tables II and III and figure 3. The yield of wheat at the last harvest was significantly reduced on soil 4, but that of corn, which showed slight symptoms of copper deficiency, was greater on soil 4 than on soils 1, 2, or 3. This observation is in harmony with the conclusion of WAYGOOD (17) that the copper containing enzyme, ascorbic acid oxidase, is the terminal oxidase of wheat plants more than one week old. Although yield is unaffected, the relative activity of ascorbic acid oxidase is also reduced in blue and yellow lupines and soybean PI-54619-5-1 on soil 4 as shown in

table I. Would this indicate that wheat alone of the plants tested has a copper containing enzyme as a dominant terminal oxidase? The copper deficiency symptoms of wheat and of some other gramineous plants are a general chlorosis in contrast with necrotic spots for many other species.

Connecticut broadleaf tobacco gave a graded response in activity of ascorbic acid oxidase on soils 1, 2, 3, and 4, the activity on soil 4 being less than 10% of that of soil 1 at all harvests as shown in figure 3. The plants growing on muck (soil 4), which has a high iron supplying capacity, did not have copper deficiency symptoms although they were as low in activity of

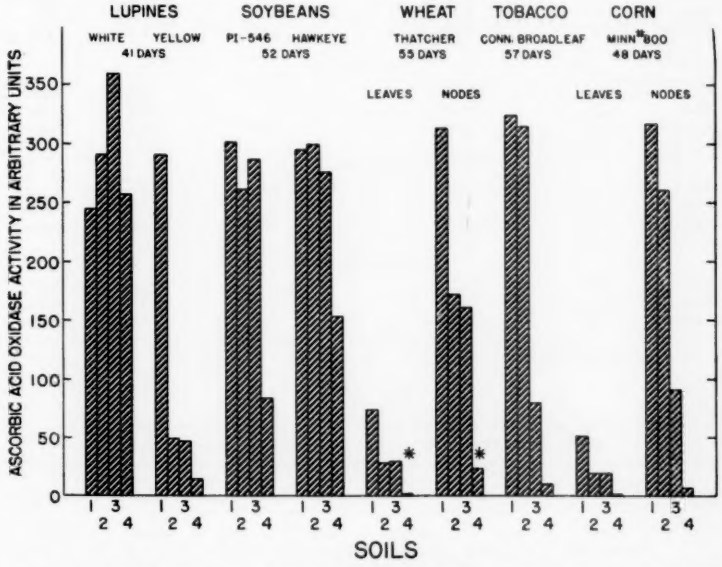

FIG. 3. Relative activity of ascorbic acid oxidase in several plants grown on soils 1 (calcareous) to 4 (organic, copper deficient). Wheat alone showed copper deficiency symptoms as indicated by the asterisk.

ascorbic acid oxidase as others which had deficiency symptoms after growth on copper deficient nutrient solutions with rather low iron supply.

Ascorbic acid oxidase activity was increased, in all plants studied, by addition of $CuSO_4$ to the organic soil. The smallest change was shown by the white lupines for which activity of ascorbic acid oxidase was high on the organic soil.

Catalase is reduced in relative activity when iron supply is limited for all plants tested except wheat and Hawkeye soybean, and the reduced activity is correlated with lower yield. In contrast, peroxidase activity varies less and without a specific pattern for a particular species as the soil is changed (tables I, II, and III and figs. 3 and 4). These observations are

TABLE I

YIELD AND RELATIVE ENZYME ACTIVITY OF LUPINES 41 DAYS OLD AND GROWN ON SOILS OF DIFFERENT IRON AND COPPER SUPPLYING CAPACITIES.

Lupine variety	Soil 1 100% Calcareous	2 66% Calcareous	33% Organic	3 33% Calcareous	66% Organic	4 100% Organic	5 100% Organic + Cu
			Yield, green wt. gm./plant				
White	3.5*	7.1		11.9		11.8	10.2
Blue	0.8*	3.0*		4.8		5.8	5.9
Yellow	1.2*	2.1*		3.0*		4.2	4.6
			Catalase (Fe)				
White	81*	348		341		226	308
Blue	52*	166*		308		365	280
Yellow	72*	69*		164*		255	223
			Peroxidase (Fe)				
White	59*	80		80		71	74
Blue	49*	28*		21		28	34
Yellow	85*	84*		82*		66	64
			Ascorbic acid oxidase (Cu)				
White	245*	291		360		257	347
Blue	338*	31*		43		17	329
Yellow	291*	49*		47*		14	223

*Fe deficiency symptoms.

TABLE II

YIELD AND RELATIVE ENZYME ACTIVITY OF WHEAT GROWN ON SOILS OF DIFFERENT IRON AND COPPER SUPPLYING CAPACITIES.

Age in days	Soil 1 100% Calcareous	2 66% Calcareous	33% Organic	3 33% Calcareous	66% Organic	4 100% Organic	5 100% Organic + Cu
			Yield, green wt. gm./10 plants				
29	29	36		41		31	35
44	63	72		82		74*	81
55	91	121		117		65*	130
			Catalase (Fe)				
29	270	313		329		185	230
44	251	244		222		156*	237
55	204	320		380		144*	394
			Peroxidase (Fe)				
29	51	61		48		47	49
44	56	65		59		64*	55
55	77	83		82		82*	82
			Ascorbic acid oxidase (Cu)				
29	10	29		9		6	15
44	58	14		12		−2*	37
55	74	28		30		−2*	38

*Cu deficiency symptoms.

well illustrated by the results for the several lupine varieties in table I and figure 4 and for corn in table III. In the yellow and blue lupines grown on soils 1 to 4, the catalase activity changes three to sevenfold with iron supply while peroxidase activity is essentially constant.

By good fortune, the changes in catalase and peroxidase activities as iron supply is varied illustrate and support the basic hypothesis that an enzyme

TABLE III

YIELD AND RELATIVE ENZYME ACTIVITY OF CORN AND SOYBEANS GROWN ON SOILS OF DIFFERENT IRON AND COPPER SUPPLYING CAPACITIES.

Crop and age	Soil 1 100% Calcareous	2 66% Calcareous 33% Organic	3 33% Calcareous 66% Organic	4 100% Organic	5 100% Organic + Cu
		Yield, gm./plant			
Corn, 34 days	25	64	72	107	124
Corn, 48 days	82*	181	232	251	393
Soybean, PI-54619-5-1	5*	17	20	32	32
Soybean, Hawkeye	8	18	21	27	31
		Catalase			
Corn, 34 days	33	88	77	72	46
Corn, 48 days	24*	62	125	193	70
Soybean, PI-54619-5-1	37*	287	292	268	186
Soybean, Hawkeye	112	138	202	119	122
		Peroxidase (Fe)			
Corn, 34 days	32	31	62	44	37
Corn, 48 days	22*	36	50	68	36
Soybean, PI-54619-5-1	30*	64	59	69	64
Soybean, Hawkeye	63	74	76	70	72
		Ascorbic acid oxidase (Cu)			
Corn, 34 days	150	61	15	16	86
Corn, 48 days	51*	19	19	−4	30
Soybean, PI-54619-5-1	302*	261	287	84	257
Soybean, Hawkeye	295	299	276	153	289

*Fe deficiency symptoms.

among those requiring the element for function is aberrant. A greater departure from normal possibly would have been shown in the activity of some other iron containing enzyme. In other words, the limiting enzyme was not necessarily found, but ones undergoing considerable change were measured.

Polyphenol oxidases were not determined, a compromise in the burden of work; but measurement of the activity of another enzyme dependent

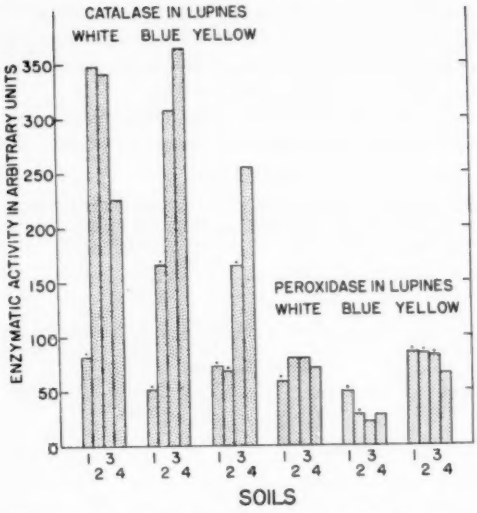

FIG. 4. Relative activity of catalase and peroxidase in three varieties of lupines grown on soils 1 (calcareous) to 4 (organic) and harvested after 41 days. Iron deficiency symptoms are indicated by the asterisk.

upon copper would have been desirable. Polyphenol oxidases are known to be absent or of very low activity in wheat (**17**) and barley (**7**). They have been suggested as being the chief terminal oxidases in cytoplasm of spinach cells (**3**) in which activity of ascorbic acid oxidase is very low.

Results for ascorbic acid oxidase, catalase, and peroxidase activities for lupines and soybeans grown on limed Chester soils (B to E) are shown in

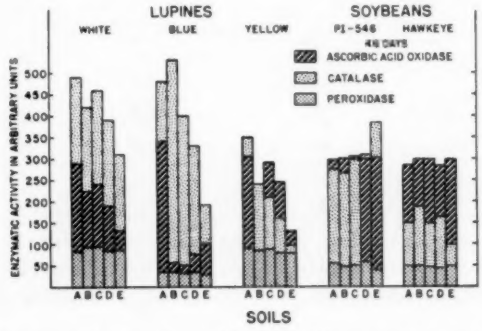

FIG. 5. Relative activities of ascorbic acid oxidase, catalase, and peroxidase, in three varieties of lupines grown on an acid soil (Chester) to which lime has been added in amounts increasing from B to E. The amount of each enzyme shown by the bar graph is to be read from the base line at zero.

figure 5. The blue and yellow lupines suffered some chlorosis without reduction in yield, and this was accompanied in each case by a relatively reduced ascorbic acid oxidase and catalase activity. The activities of the three enzymes measured were relatively constant for the two soybean varieties on the limed soils.

Variation of an enzymatic activity pattern with the nutritional level of a crop would appear to offer far greater likelihood of success in following plant nutrition than has been possible by other methods. One of the few examples in the literature of this principle are the reports of reduced activity of aldolase in zinc deficient yeast, oats, and clover (**4, 12**).

Summary

Relative activities of ascorbic acid oxidase, catalase, and peroxidase were measured at two or three periods of growth for one variety each of corn, wheat, and tobacco, three species of lupines, and two varieties of soybeans grown on a soil of limited copper supplying capacity, another of limited iron supplying capacity, and two mixtures of these soils.

Ascorbic acid oxidase activity is markedly reduced by limited copper supply and is correlated with a reduction in yield for wheat in which it is a terminal oxidase.

Catalase was reduced in relative activity for corn, tobacco, the lupines, and one variety of soybean when the iron supply was limited. The activity of peroxidase was more constant.

The hypothesis that if an element is limiting in the nutrition of a plant the deficiency will be evident in the changed activity of an enzyme among those requiring the element for function is in harmony with the results obtained.

The authors are indebted to Dr. J. F. Davis and R. Gillispie, Michigan State College Experimental Muck Farm, East Lansing, Michigan, for the organic soil, to Dr. D. W. Thorne, Utah State Agricultural College, Logan, Utah, for a calcareous Millville silt loam, to Dr. M. G. Weiss for seed of soybean PI-54619-5-1 used in these experiments, and to Dr. R. A. Steinberg for tobacco grown on nutrient solutions.

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LONGITUDINAL GRADIENTS OF P^{32} ABSORPTION IN ROOTS

PAUL J. KRAMER AND HERMAN H. WIEBE

(WITH FIVE FIGURES)

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It is generally agreed that the greatest accumulation of ions occurs in the cells near the tips of the roots. PREVOT and STEWARD (**17**) observed that accumulation of bromide ions by excised barley roots was greatest in the apical centimeter; and STEWARD *et al.* (**20**) found that accumulation of potassium by roots of intact barley seedlings and of rubidium by excised roots 5 cm. in length increased progressively toward the tip and was greatest in the shortest segments analyzed (0.5 cm.). Experiments of OVERSTREET and JACOBSON (**16**) indicated that at 0° C maximum absorption of radioactive rubidium and phosphorus by apical segments of barley roots 1 cm. in length occurs within a millimeter of the tip. JACOBSON and OVERSTREET (**7**) later reported that maximum absorption of radioactive iodine and strontium occurs within 2 or 3 mm. of the root tip. STEWARD (**19**) has described in summary form work carried out in his laboratory in conjunction with Drs. R. Overstreet and S. M. Caplin. The accumulation of Cs^{137} in roots was studied by means of autoradiograms and by measuring the radioactivity of 1-mm. segments at intervals of 1 or 2 mm. from the apex to 10 cm. behind the apex. The published autoradiograms and counts show puzzling irregularities in accumulation along a single root and differences between roots from the same plant that could not be related to structure or physiological condition of the tissues.

While studying P^{32} absorption of pine roots, the authors obtained a number of autoradiograms. These showed that, as expected, there was usually heavy accumulation in the root tips and lower accumulation a few millimeters behind the root tip. Unexpectedly, however, a number of roots showed other regions of high accumulation extending some distance behind the tips. A typical example of this accumulation was shown in figure 2a of KRAMER and WILBUR (**9**). Since many examples of this unexpected pattern of accumulation were observed, further study was made to find how extensively it occurs. Efforts were also made to find what relations exist between dry weight, total nitrogen content, total phosphorus content, and radioactive phosphorus content of root segments at various distances behind the root apex.

Experiments and results

The pine roots used in this study were obtained from loblolly pine seedlings growing in pots in a cold frame. The tomato roots were adventitious roots produced on cuttings grown in aerated Hoagland's solution containing

no phosphorus. The barley roots were from seedlings germinated in half-strength Hoagland's solution lacking phosphorus.

Autoradiograms

For preparation of autoradiograms root segments several centimeters in length were carefully freed of all foreign matter and immersed for one and one half to three hours in solutions of P^{32} having an activity of 200 to 300 μc. per liter. This solution was kept in shallow finger bowls at room temperature; and since considerable surface was exposed to the air and the liquid was quite shallow, no forced aeration was considered necessary. Most of the roots were detached from the plants, but the barley roots were left attached. At the end of the immersion period, the roots were washed in several changes of distilled water, rinsed in 0.01 N H_3PO_4, and quickly dried in an oven between layers of glass cloth. Glass cloth treated with Desicote (an anti-wetting agent) was used because root tips do not stick to it as they do to most materials. After the roots were dry, they were laid on a glass plate, or in later experiments on thick blotting paper, and covered with a sheet of aluminum foil or Pliofilm such as is sold in grocery stores. The photographic plate was brought into firm and uniform contact with the foil or plastic by means of rubber bands or by weights. The aluminum or plastic was inserted between the root and the plate to prevent any possibility of chemical action on the emulsion. The roots were left in contact with the photographic plates for periods varying from two to three hours to several days. The autoradiograms were made on Kodak M plates, except figure 1 A, which was made on a medium lantern slide plate.

Autoradiograms of a number of roots are shown in figure 1. It is quite apparent that considerable variation exists in distribution of radioactive phosphorus in different roots, even in those of the same species grown under identical conditions. Figure 1 A shows autoradiograms of two large roots of red pine (*Pinus resinosa* Ait.). They exhibit high accumulation of P^{32} in the tips. There is a well defined region of low accumulation beginning 2 or 3 mm. behind the apex which probably is in the region of rapid cell enlargement. The localized regions of high accumulation on the older parts of the roots are branch roots, either mycorrhizal or non-mycorrhizal. The four autoradiograms of figure 1 B are from small loblolly pine roots (*Pinus taeda* L.). The first and fourth roots show the same accumulation pattern as the red pine roots, but the second and the third roots show little accumulation in the tips. All four show heavy accumulation a centimeter or more behind the root tips.

All four of the autoradiograms of adventitious roots of tomato reproduced in figure 1 C exhibit high accumulation of P^{32} in the meristematic region, but their accumulation patterns are otherwise quite dissimilar. There was negligible accumulation in most of the older portion of the first root; noticeable accumulation occurred only in the first centimeter of the second root; but heavy accumulation occurred along the entire length of

the third root, except in the region of elongation. The fourth root showed variations in P^{32} accumulation in the older portion which cannot be related to root structure.

The autoradiograms of barley roots in figures 1 D and 1 E also show

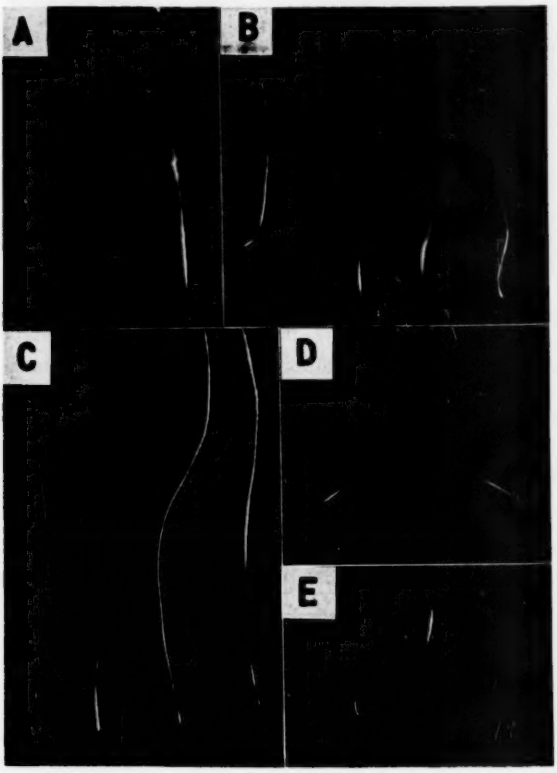

FIG. 1. Autoradiograms of roots of four species showing variations in absorption of P^{32}. A. Two large roots of red pine. B. Four small roots of loblolly pine. C. Four adventitious roots of tomato. D and E. Seedling roots of barley. Most autoradiograms show heavy absorption in the meristematic region, lower absorption in the region of elongation, and heavy absorption a centimeter or more behind the root tip. Many exceptions to this pattern occur, however, as shown by these autoradiograms, of which A and D might be regarded as typical while B, C, and E show variations from the typical pattern.

variability in respect to accumulation of P^{32}. The three roots of figure 1 D are similar in having high accumulation in the root tip, lower accumulation in the region of elongation, and very heavy accumulation a centimeter or more behind the apex where root hairs are well developed. The three roots

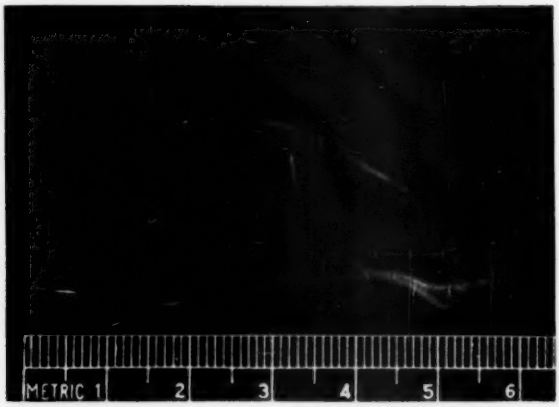

FIG. 2. Autoradiograms of barley roots, showing uneven distribution of P^{32}. The three roots originated from a single seed.

of figure 1 E show little accumulation in the tip and an irregular pattern of accumulation in the older parts of the roots.

Figure 2 is an enlargement of the autoradiograms of three barley roots produced from the same seed. The enlargements show a surprisingly irregular distribution of P^{32}, with alternating regions of high and low concentration. Comparison of the autoradiograms with the activity of 2-mm. segments cut from the same root, as measured by counting the individual segments, shows good general agreement between regions of high and low concentration as measured by the two methods (fig. 3) which seems to

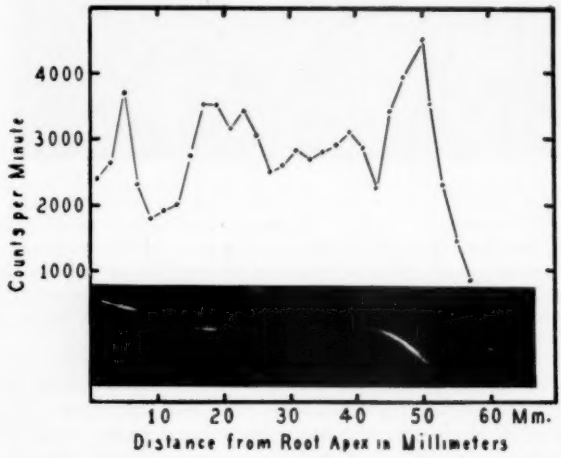

FIG. 3. Autoradiograms of a barley root, with concentration of P^{32} as determined by counting 2-mm. segments graphed above it.

eliminate any possibility that the various irregularities in distribution of P^{32} shown in the autoradiograms are artifacts produced during their preparation. Such artifacts might result from differences in diameter of the roots, differences in amount of flattening during drying, from bends in the roots, or from lack of uniform contact between the roots and the photographic plates.

Activity measurements

It is difficult to determine accurately small differences in relative activity from autoradiograms. Roots therefore were dried as previously described, cut into short segments, and the activity of each segment measured indi-

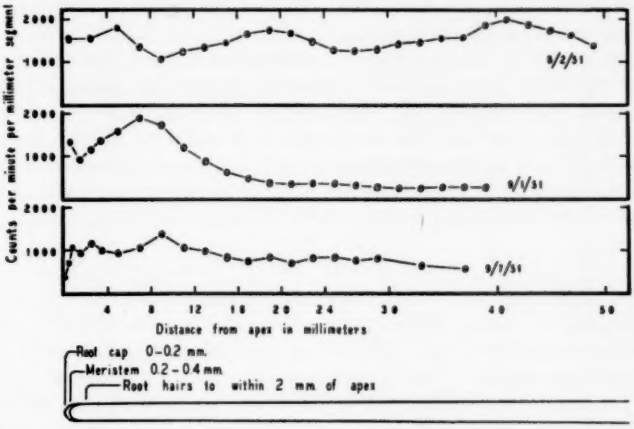

Fig. 4. Graphs showing the amount of P^{32} absorbed by various regions of barley roots, expressed as counts per minute per millimeter segment, also a diagram showing some features of barley root anatomy. The roots were cut into segments 2 mm. in length for counting, except at the tips where the segments were 1.0 mm. long for the series of September 1 and 0.2 mm. for that of September 7. The curve for August 2 is the average of three roots, that for September 1 the average of six roots, that for September 7 the average of four roots.

vidually with a Geiger-Muller counter. For this purpose the older portions of the roots were cut into 2-mm. segments, but the tips were cut into segments only 1.0 or 0.2 mm. in length to permit more accurate localization of the regions of high and low absorption. The averages of three sets of such measurements are summarized graphically in figure 4. Below the counts is shown a diagram indicating the approximate location of various regions of a barley root. This diagram is based on observations of the experimental roots and agrees in general with results of a detailed study of barley root anatomy recently published by Heimsch (**6**).

While the location of various structural regions of the barley roots varied but little, the autoradiograms and counts of activity shown graphically in figure 4 indicate that the pattern of P^{32} concentration varies appreciably.

The root tips of the experiment of August 2, 1951 (fig. 4), showed high accumulation throughout the first 50 mm. with regions of high accumulation 5, 18, and 42 mm. behind the tip. In the experiment of September 1, 1951, maximum accumulation was 9 mm. behind the tip, and in that of September 7, 1951, there was a region of high accumulation at 1 to 3 mm. and another at 9 mm. from the tips. The series of September 7 was the only one in which the root cap was counted separately, and in this series very little accumulation was found in the root cap. Differences in location of the region of heavy accumulation near the tip might possibly be related to differences in rate of growth of the roots immediately preceding and during the period of exposure to P^{32}. It seems that their past history has considerable effect on the accumulation pattern of the roots, and this probably accounts for the differences among the three groups of roots.

The results of the experiments indicate that the heaviest accumulation of P^{32} by barley roots generally occurs in the root hair zone. There is a suggestion that variations in accumulation along the older regions of the roots is related to variations in number and condition of root hairs. In pines, root hairs are less common and there is a tendency for the epidermal cells to slough off. Nevertheless, in pine, as in barley, maximum accumulation of P^{32} often occurs some distance behind the apical meristem.

Chemical analyses

An effort was made to see if any correlation existed between chemical composition of various regions of the root and the amount of P^{32} accumulated by cutting roots into segments and analyzing them for total nitrogen and total phosphorus. For the analyses the apical centimeter of each root was cut into 2-mm. segments, a segment was removed 20 mm. behind the apex and another about 50 mm. behind the apex. The corresponding segments of 4 to 10 pine roots, depending on their size, and 50 barley roots were pooled to provide samples large enough for analysis.

Fresh weight and oven-dry weight of the samples were obtained by weighing on a micro balance. For analysis of total nitrogen and total phosphorus, the samples were digested in hot sulphuric acid and cleared with 30% hydrogen peroxide, then diluted to 5 ml. and aliquots removed. Total nitrogen and total phosphorus were determined colorimetrically by essentially the same methods described by Lindner (10).

The results of the analyses of barley roots are summarized in figure 5. The apical segments of the barley roots, which included the root cap and the meristematic region, were low in fresh weight, relatively high in dry weight, and, although smaller in volume, contained about twice as much total nitrogen and total phosphorus per segment as any other part of the root. The fresh weight increased basally, as would be expected, because of an increase in water content in the region of cell enlargement and the increase in dry matter in the region of differentiation. The high dry weight and high nitrogen and phosphorus contents of the apical segment conform

with the view that it consists largely of protoplasm in which the vacuoles occupy a relatively small volume. The abrupt decrease in dry weight behind the apical segment doubtless is the result of the rapid enlargement and increased volume of vacuoles of cells in this region, accompanied by

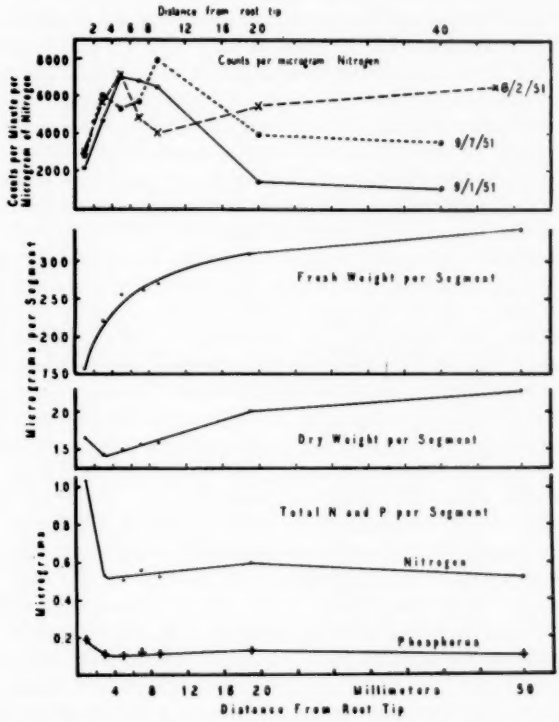

FIG. 5. Analyses of barley roots. The data for fresh and dry weight, total nitrogen, and total phosphorus represent the averages of five groups of 50 roots each. The tip centimeter was cut into segments 2 mm. in length, a 2-mm. segment was cut 18 to 20 mm. behind the tips, and another 48 to 50 mm. from the tips. The upper graph shows the amount of P^{32} absorbed per microgram of nitrogen for each 2-mm. segment. Although considerable variation occurred in the amount of P^{32} absorbed by the older portions of the roots, all three groups show low absorption per unit of nitrogen in the meristematic region and high absorption 3 to 10 mm. behind the tip. Compare with figure 4, showing uptake of P^{32} per segment by the same groups of roots.

little increase in dry matter. Further back, cell walls become thickened and dry weight increases. Both total nitrogen and total phosphorus show little change per segment behind the apex. This observation suggests that little new protoplasm is synthesized in the region of these roots 5 to 50 mm. from the apex and the increase in dry weight must be largely cell wall material.

The analyses of pine roots showed essentially the same gradients in all constituents, except for a decrease in fresh weight in the 48 to 50-mm. segments, caused by suberization and death of epidermal and cortical cells in this region. The curves showing the amount of P^{32} accumulated per microgram of nitrogen will be discussed later.

These analytical results are in general agreement with those reported for onion roots by NORBERG (**14**) and NORRIS (**15**). BROWN and BROADBENT (**4**) measured the dry weight, protein nitrogen, cell number, and cell volume in pea roots. When measured on a segment basis, dry weight and protein nitrogen were highest 2 mm. from the tip and decreased in segments 4 and 5 mm. behind the tip. On a cell basis, however, dry weight increased rapidly from the apex to 5 mm., then increased gradually, while protein nitrogen reached a maximum at 5 mm. from the apex and decreased slightly in segments from 5 to 10 mm. from the apex.

The meristematic region generally is regarded as a region of high metabolic activity. According to MACHLIS (**13**) (barley roots) and BERRY and BROCK (**3**) (onion roots) root respiration per segment and per unit of volume is highest near the apex. In corn roots, according to GODDARD and MEEUSE (**5**), most active respiration on a fresh weight basis occurs in the apical millimeter; but if based on total nitrogen, protein, or ribose nucleic acid content, respiration is lowest in the apical 1 or 2 mm. and most rapid respiration occurs 4 to 5 mm. behind the tip where cells are elongating. Brown and Broadbent found respiration per segment of pea roots to be highest at 1.2 mm. behind the apex and respiration per cell to be highest at about 5 mm. behind the apex, decreasing somewhat in cells between 5 mm. and 9 mm. behind the apex where measurement stopped. If respiration is calculated in terms of total nitrogen, as is often done, the high nitrogen content of the barley root tips modifies the respiration curve. When the oxygen consumption of barley roots, as measured by MACHLIS (**13**), is calculated in terms of the total nitrogen content of barley roots used in this investigation, it is slightly, but probably not significantly, higher in the 20-mm. region than in the apical region. BROWN and BROADBENT (**4**) found a close correlation between protein nitrogen and respiration in pea roots, except possibly for the apical 0.4-mm. segment, which appeared to have a high rate per unit of protein nitrogen.

BERRY and BROCK (**3**) suggested that the high oxygen consumption of root tips does not necessarily indicate a higher rate of respiration per unit of material but probably is merely the result of a larger quantity of respiring material per segment. This view seems to be supported by the data presented here and also by those of NORRIS (**15**), who reported that if respiration of onion root segments is calculated per unit of nitrogen, it is no higher in the apical 5 mm. of the root than in segments farther back. He did find a gradient if the rate of respiration was calculated in terms of phosphorus content, but this would not be true of the data of this investigation.

BERRY and BROCK (3) found respiration of the apical 5-mm. segment of onion roots to be inhibited more by cyanide than the respiration of older segments. This inhibition suggests that the meristematic region has a different type of oxidative metabolism from that found in older portions of the root. BERRY (2) found that respiration of the apical 5-mm. segments of onion roots is limited by oxygen, which is not true of older portions of the root.

The amount of P^{32} accumulated per segment of barley root in this study was calculated in terms of total nitrogen per segment, and the results are shown in the upper part of figure 5. As would be expected from the data presented in figure 4 and those for total nitrogen in figure 5, the concentration of P^{32} per microgram of nitrogen is lower in the apical segment than anywhere else in the terminal 50-mm. portion of the roots. These relationships are shown numerically in table I where it can be seen that the ratio of phosphorus to total nitrogen is not significantly different in the various root

TABLE I

RELATION BETWEEN TOTAL NITROGEN, TOTAL PHOSPHORUS AND RADIOACTIVE PHOSPHORUS IN SEGMENTS OF BARLEY ROOTS.

Distance from tip	Total N per root segment	Total P per root segment	Ratio total P to total N	Counts per minute per root segment	Ratio P^{32} to total P × .001
mm.	*μg.*	*μg.*			
0 to 2	.514	.0985	.192	1360	13.8
2 to 4	.269	.0535	.199	1470	27.5
4 to 6	.255	.0515	.202	1650	32.1
6 to 8	.279	.0540	.194	1610	29.9
8 to 10	.264	.0570	.216	1620	28.5
18 to 20	.294	.0625	.212	1070	17.1
48 to 50	.260	.0500	.192	965	19.3

segments. The ratio of radioactive phosphorus to total phosphorus is only half as great in the apical segment as it is in segments located 2 to 10 mm. behind the root tip. Evidently accumulation of radioactive phosphorus is not directly correlated with the concentration of total phosphorus. Neither is it directly correlated with the synthesis of protein which presumably is occurring most rapidly in the meristematic region located in the apical segment.

In the roots studied, accumulation of P^{32} seemed to be greater per unit of nitrogen in cells which are enlarging and differentiating than in cells which are dividing. Accumulation also was high, at least in some instances, in epidermal cells which are not even capable of differentiation. SANDSTROM (18) suggested that selective absorption of ions is localized in the epidermis. While the methods employed did not permit exact localization, autoradiograms of cross-sections of roots show the P^{32} to be concentrated near the surface; and none could be found in the central part of the apical meristem. This unexpected situation is being investigated further.

Discussion

In these experiments, the initial activity of P^{32} in the external solution was usually 200 to 300 μc. per liter, the final activity considerably lower. In one experiment subjected to careful analysis, the final activity of the external solution was 280 μc. per liter, equivalent to a concentration of 3.1×10^{-11} moles of P^{32} per liter. The total phosphorus concentration of the solution was 8.1×10^{-7} moles per liter and the specific activity was therefore 3.8×10^{-5}. Specific activity is the concentration of P^{32} divided by the concentration of total phosphorus. After two hours in this solution, the P^{32} concentration in the roots on a volume basis was 1.2×10^{-8} moles per liter. Thus, the concentration of P^{32} in the roots was approximately 400 times as great as that of the external solution. The specific activity of P^{32} in the roots was only 1.1×10^{-6} moles per liter, or considerably less than in the solution. Since the amount of P^{32} in the roots is so small in proportion to the total phosphorus present, some question has arisen whether the uptake of P^{32} observed in these experiments can be regarded as true metabolic accumulation. Uptake of P^{32} might occur as the result of metabolic accumulation, it might occur as a result of exchange with phosphorus already present in organic compounds in the root, or by the non-metabolic exchange absorption of OVERSTREET and JACOBSON (**16**). The phosphorus might be precipitated as an iron or calcium salt on the surface of the root, or it might be absorbed in the meristematic region and then translocated upward. Perhaps all of these processes occur to a limited extent, but it is believed that most of them are of negligible importance in this study.

The possibility of upward translocation from the root tip to the root hair zone was studied by supplying P^{32} to restricted areas on barley roots through potometers. When P^{32} was supplied to the root tips for 10 hours, little upward translocation occurred. When it was supplied to the root hair zone, considerable translocation occurred downward to the tip of that root, upward to the seed and leaf, and even down into the tips of other roots of the seedling. It seems certain that upward translocation of P^{32} from the meristematic region cannot account for the large amounts of P^{32} found in the root hair zone, and it must have been for the most part absorbed in the regions where it was later found to be present.

In all of our experiments, the roots were washed in dilute acid, usually 0.01 *N* H_3PO_4, but in some instances in 0.005 *N* HCl, then rinsed in distilled water. This acid wash should have removed any P^{32} precipitated on the surface of the roots. Actually such washing removes little P^{32} and does not change the pattern of the autoradiograms.

There is little doubt that the major part of the P^{32} uptake observed in these experiments is controlled by metabolic activity. The uptake of P^{32} is about 10 times as great at 20° as at 3° C in both barley and pine roots. Perhaps the rate at 3° C is a measure of non-metabolic absorption. KRAMER (**8**) found that azide and fluoride at a pH of 4.7 to 4.8 reduced accumulation

of P^{32} by pine roots to less than 5% of the controls. He also found that dead pine roots absorbed less than 5% as much P^{32} as living roots. It seems that the possibilities of appreciable surface precipitation or translocation have been eliminated, and the amount of P^{32} accumulated is much greater than can be accounted for by non-metabolic absorption. Possibly exchange between P^{32} in the external solution and P^{31} in the cells is controlled by metabolic activity, but this seems improbable. Exchange with P^{31} existing in organic compounds within the cells should be greatest in the meristematic region where the highest concentration of P^{31} occurs and metabolic activity is rapid, but as shown in table I and figure 4 uptake of P^{32} is lower in this region than further back where the concentration of P^{31} is lower.

In tracer studies, it is difficult to prove that the path and manner of uptake of the radioactive isotope is an accurate indicator of the path and manner of uptake of the non-radioactive isotope. Such a similarity has not been proven in this study, but it has been shown that uptake of P^{32} is reduced by treatments which are known to reduce accumulation of ions. While more evidence would be desirable, it seems probable that the uptake of P^{32} observed in these experiments was really metabolic accumulation and probably is indicative of the manner in which phosphorus usually is absorbed.

It seems clear from the results of these studies that accumulation of P^{32} is not restricted to the meristematic region of roots but often occurs several centimeters behind the root tip. In contrast the non-metabolic absorption observed by OVERSTREET and JACOBSON (**16**) at 0° C is highest in the meristematic region. We have also observed highest accumulation in the meristematic regions of roots exposed to P^{32} at 3° C. JACOBSON and OVERSTREET (**7, 16**) question the role of root hairs in mineral absorption because in their experiments dealing with non-metabolic absorption with 1-cm. segments at 0° C most of the uptake occurred below the root hair zone. LUNDEGARDH (**11, 12**) reported that wheat seedlings with few root hairs absorb less salt than those with numerous root hairs. He regards root hairs as quite important in the absorption of ions and states that in wheat roots maximum absorption occurs 10 to 25 mm. behind the tip, where root hairs are well developed. There seems to be little doubt that extensive accumulation of P^{32} occurs in the root hair zone of both barley and tomato roots, and this P^{32} is translocated more readily than that accumulated in the meristematic region of roots.

It is difficult to explain variation in the region of maximum uptake of P^{32} among roots of identical past treatment. As STEWARD (**19**) stated with respect to Narcissus roots, these variations are not always explainable in terms of obvious features of root anatomy or development. Steward observed alternations in regions of high and low concentrations of Cs^{137} similar to those shown in figure 2 for P^{32}. Variations in growth during the preceding two or three days might produce differences in number and condition of root hairs and permeability of epidermal cells. LUNDEGARDH (**11**) mentioned the difficulty of producing uniform populations of roots and root

hairs, and OVERSTREET and JACOBSON (16) mentioned individual variations among barley roots. While the past history might explain variations in amount of salt accumulated and in regions of accumulation, it does not explain the bands of high and low concentration visible in figure 2.

It is generally supposed that salt accumulation occurs chiefly in cells which are actively growing, or at least in cells which are capable of growth and differentiation. While this probably is generally true, some exceptions were observed. Not only does appreciable uptake of P^{32} occur in regions of barley, pine, and tomato roots where little or no growth is occurring, but it also occurs in epidermal cells of pine which are sloughing off. In pine as described by ADDOMS (1) the epidermis near the root tip often begins to split off in long strips which remain alive for several days. Although such strips occasionally show limited elongation, they do not divide or differentiate and finally die; yet they absorb P^{32}. It may be debatable as to whether or not this is true accumulation, but the P^{32} activity of the tissue becomes several hundred times greater than that of the surrounding solution.

In conclusion, it appears that study of uptake of radioactive ions by individual roots is revealing individual differences not detectable by mass analysis of large numbers of roots. The available data indicate that there are much greater variations among roots of similar past history in respect to respiration and salt uptake than with respect to structure. While these variations complicate our studies, they may eventually add materially to our understanding of salt absorption.

Summary

Detached roots of pine and tomato and attached roots of barley were exposed to P^{32} solutions having an activity of 200 to 300 μc. per liter for one and one half to three hours at 22 to 28° C, and autoradiograms were made. Some of the roots were then cut into segments 0.2 to 2.0 mm. in length, and the activity of individual segments was measured with a Geiger-Muller counter. There was good agreement between the relative activity of segments of barley roots measured with a counter and their activity as indicated by the autoradiograms.

Considerable variation in distribution of P^{32} was found, even in roots grown under identical conditions. Some roots showed high uptake of P^{32} only at the tip; others showed little or no uptake at the tip but considerable uptake farther back. The most common condition was relatively high concentration in the root tip, a region of lower concentration a few mm. behind the tip which seemed to coincide with the region of elongation, and another region of high concentration 6 to 20 mm. behind the tip.

Absorption of P^{32} was not restricted to the meristematic region of the roots of any species studied. Heavy uptake occurred in the root hair zone of many barley and tomato roots and in epidermal cells of pine one or more centimeters behind the root tip.

Five groups of week-old barley roots about 50 mm. in length were cut into 2-mm. segments; and the fresh weight, dry weight, total nitrogen, and

total phosphorus content of each group was determined. The apical segments were low in fresh weight, relatively high in dry weight, and contained twice as much nitrogen and phosphorus per segment as any other region of the roots. Fresh weight increased basally from the apex, but the dry weight per segment was lower in segments 2 to 6 mm. behind the apex than in the segment 0 to 0.2 mm. from the apex. The dry weight increased gradually toward the base. Loblolly pine roots showed similar gradients in all constituents.

The uptake of P^{32} per segment was calculated in terms of total nitrogen content per segment and was found to be much lower in the meristematic region than in segments one or two centimeters behind the root tip. The highest uptake per unit of nitrogen appeared to be in the region where differentiation was occurring.

Although other processes may be involved, the uptake of P^{32} observed in this study is believed to represent principally metabolic accumulation because uptake is reduced to less than 10% of control by respiration inhibitors and by low temperatures.

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THE DISTRIBUTION IN HIGHER PLANTS OF TRIPHOSPHOPYRIDINE NUCLEOTIDE-LINKED ENZYME SYSTEMS CAPABLE OF REDUCING GLUTATHIONE

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The enzyme glutathione reductase, which catalyzes the reduction of glutathione (GSSG) by triphosphopyridine nucleotide (TPNH) according to equation 1, was

(1) $$TPNH + H^+ + GSSG \longrightarrow 2\ GSH + TPN^+$$

demonstrated independently and simultaneously by MAPSON and GODDARD (15) in peas, and by CONN and VENNESLAND (5) in wheat germ. The work described in the present paper was undertaken initially with the object of determining the distribution of this enzyme in the tissues of higher plants.

The enzymic reduction of GSSG can be coupled with enzyme systems capable of reducing TPN. A procedure for assaying the glutathione reductase of wheat germ depends on such coupling with glucose-6-phosphate dehydrogenase, which catalyzes the reaction of equation 2.

(2) $$\text{glucose-6-phosphate} + H_2O + TPN^+ \longrightarrow \text{6-phosphogluconate} + TPNH + 2H^+$$

The method involves the determination of the amount of GSH formed after incubation of GSSG, TPN, glucose-6-phosphate and an excess of glucose-6-phosphate dehydrogenase with the material to be tested. When these enzymes and substrates are present together, the net reaction catalyzed is that shown in equation 3. Only catalytic amounts of TPN are required.

(3) $$GSSG + H_2O + \text{glucose-6-phosphate} \xrightarrow{TPN} 2\ GSH + \text{6-phosphogluconate} + H^+$$

Any other dehydrogenase system requiring TPN may be substituted for the glucose-6-phosphate dehydrogenase system to give analogous coupled reactions. Once the reductase has been shown to be present in a particular extract, it is possible to demonstrate the presence of any TPN-linked dehydrogenase in that extract by determining whether the substrate of the dehydrogenase will reduce GSSG. Thus, the isocitric dehydrogenase system coupled with glutathione reductase, has been shown to catalyze the reaction given in equation 4; and the malic enzyme system with glutathione reductase, gives the reaction of equation 5 (15). The results of the application of such tests to a variety of plant extracts are described in the present paper.

(4) $$\text{isocitrate} + GSSG + H^+ \xrightarrow{TPN,Mn^{++}} 2\ GSH + \alpha\text{-ketoglutarate} + CO_2$$

(5) $$\text{malate} + GSSG + H^+ \xrightarrow{TPN,Mn^{++}} 2\ GSH + \text{pyruvate} + CO_2$$

Materials and methods

Triphosphopyridine nucleotide of 70% purity was generously supplied by Dr. M. A. Mitz of Armour Laboratories. Glucose-6-phosphate was prepared by the method of LEVENE and RAYMOND (**14**). Glucose-6-phosphate dehydrogenase was prepared according to KORNBERG (**12**). Glutathione was prepared from GSH (Schwarz) by oxidation with H_2O_2 (**17**). L-malic acid (Eastman) was recrystallized three times from ethyl acetate-petroleum ether and *dl*-isocitric acid was prepared by the method of FITTIG and MILLER (**9, 13, 16, 18**) and contained less than 5% of the lactone. By enzymic assay, 45 to 50% of the material was *d*-isocitric acid (**2**).

Tris(hydroxymethyl)aminomethane (tris) was purchased from Commercial Solvents Corporation and recrystallized from ethanol or an acetone-water mixture. Collidine (2,4,6-trimethylpyridine) was obtained from the Matheson Company. The fraction boiling in the range 169.5 to 171° (uncorrected) was used. Buffers were made by adjusting the pH of solutions of tris or collidine with HCl. In the case of collidine buffers, insoluble material was allowed to settle out and was discarded.

Wheat germ extract was prepared by a previously published method (**5**). Extracts of leafy tissues were prepared by grinding the frozen leaves in a mortar. The melted slurry was strained through cheese cloth. Carrot root, sweet potato root, potato tuber, and avocado fruit were peeled, sliced, frozen and treated in the same manner. In the case of avocado, water was added to the slurry and the solids were separated by centrifugation. Parsley root and cucumber and cantaloupe fruits were peeled and ground in a meat grinder. The juice was strained through cheese cloth. An extract of *Sedum spectabile* was prepared by grinding frozen leaves with an equal volume of cold 0.5 M phosphate buffer, pH 7.4. The extract set to a gel, which underwent syneresis on standing in the cold. The liquid exuded from the gel was used. Etiolated pea seedlings three days old were homogenized for one minute at 0° C in the Waring Blendor with twice their volume of cold 0.1 M phosphate buffer, pH 7.1. All the above extracts were centrifuged at 22,000 × g for 10 to 30 minutes. The supernatant fluid was dialyzed for 24 hours in the cold against 0.025 M phosphate buffer (pH 7.4). Dry weights of the extracts were determined by drying a measured volume of the extract at 110° C for 16 hours and correcting for buffer salts present.

The composition of the various reaction mixtures is described in tables I, II, and III. The reaction was started by adding the plant extract to the rest of the mixture, both solutions having been brought separately to 30° C. After incubation for the period noted on the tables, the reaction was stopped by adding 0.3 ml. of 20% metaphosphoric acid. The protein precipitate was centrifuged and an aliquot of the supernatant fluid was titrated at 0° C with 0.001 *N* potassium acid iodate, according to the method of FUJITA and NUMATA (**10**).

Results

Glutathione reductase and glucose-6-phosphate dehydrogenase

The distribution of glutathione reductase in plant tissues was determined by coupling this enzyme with the glucose-6-phosphate dehydrogenase system. Glucose-6-phosphate dehydrogenase, prepared from yeast, contained little or no glutathione reductase activity. Various preparations differed somewhat in this regard. In one, glutathione reductase could not be detected; in two others, small amounts of the reductase were found, and appropriate corrections were applied to the results when these preparations were used. These corrections were of the order of 0.50 micromole of GSH formed in 45 minutes. The dialyzed plant extract was incubated with

TABLE I

DISTRIBUTION OF GLUTATHIONE REDUCTASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN PLANT TISSUES.

Tissue	Incubation period	Milliliters of 0.001 *N* $KIO_3 \cdot HIO_3$ (micromoles GSH formed)				
		Complete system	Glucose-6-phosphate dehydrogenase omitted	Glucose-6-phosphate omitted	TPN omitted	GSSG omitted
	min.					
Wheat germ	10	2.43	2.43	0.06	0.06	0.06
Spinach leaf	10	2.64	1.10	0.08	0.08	0.06
Sunflower leaf	45	1.50	0.14	0.14	0.11	0.11
Tomato leaf and stem	30	0.84	0.39	0.15	0.06	0.06
Cabbage leaf	30	1.08	0.95	0.17	0.07	0.06
Carrot root	30	3.18	0.84	0.54	0.06	0.12
Sweet potato root	60	0.95	0.69	0.17	0.06	0.08
Potato tuber	30	1.30	1.19	0.48	0.12	0.35
Avocado fruit	15	1.41	1.41	0.12	0.12	0.12
Cantaloupe fruit	45	4.29	2.99	0.44	0.39	0.04

GSSG, TPN, glucose-6-phosphate and glucose-6-phosphate dehydrogenase, and the amount of GSH formed was determined. This amount was compared with the GSH titer obtained when each of the components of the system was omitted separately from the incubation mixture. The conditions which were used are optimal for the coupled systems in wheat germ extracts and are given in tables I and II. The complete system for table I consisted of glucose-6-phosphate dehydrogenase, 125 micrograms; glucose-6-phosphate, 3.7×10^{-3} M; GSSG, 6×10^{-4} M; TPN, 1×10^{-5} M; tris buffer, pH 7.4, 3.3×10^{-2} M; and plant enzyme preparation. Three micromoles of adenosine-5-phosphate were added to the reaction mixtures with sunflower leaf and sweet potato root. In the reaction mixtures with sunflower leaf, tomato

leaf and stem, and sweet potato root, the concentration of glucose-6-phosphate was 1.8×10^{-3} M and that of GSSG was 1.2×10^{-3} M. The total volume was 2.7 ml. and the temperature was 30° C.

The complete system of table II consisted of glucose-6-phosphate, 1.8×10^{-3} M; GSSG, 7.8×10^{-4} M; TPN, 1×10^{-5} M; tris buffer, pH 7.4, 3.3×10^{-2} M; and plant enzyme preparation. The volume was 2.7 ml., and the mixtures were incubated for 45 minutes at 30° C. The amount of glucose-6-phosphate dehydrogenase added was 125 micrograms.

In table I, glucose-6-phosphate dehydrogenase was added to all incubation tubes except one. In table II, the dehydrogenase was added to only one incubation tube. This variation in procedure causes no significant difference in the results. One fact demonstrated by the data of table I and table II is that all the plant sources tested contained glutathione reductase.

TABLE II

DISTRIBUTION OF GLUTATHIONE REDUCTASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN PLANT TISSUES.

Tissue	Milliliters of 0.001 *N* $KIO_3 \cdot HIO_3$ (micromoles GSH formed)				
	Complete system	Complete system glucose-6-phosphate dehydrogenase added	Glucose-6-phosphate omitted	TPN omitted	GSSG omitted
Etiolated pea seedling	2.53	2.38	0.15	0.11	0.21
Sedum leaf	1.66	1.65	0.24	0.14	0.12
Parsley leaf and stem	1.77	1.90	0.14	0.12	0.12
Parsley root	0.56	2.19	0.11	0.12	0.11
Cucumber fruit	2.11	2.11	0.11	0.12	0.11

The figures for the complete system in table I and the mixture with glucose-6-phosphate dehydrogenase in table II show the amount of GSSG reduction obtained when glucose-6-phosphate dehydrogenase is not limiting. In the case of sunflower leaf and sweet potato, negative results were obtained unless adenosine-5-phosphate was added to the test system. A possible interpretation of these findings is that the adenylic acid protects TPN against destruction (**6**).

The quantity of extract to be employed was determined empirically. In general, 0.5 to 1.0 ml. of extract was tested in a preliminary experiment; and if the results were not regarded as conclusive, larger and smaller quantities were tested. It should be emphasized that in experiments of this sort larger amounts of extract may give rise to smaller rather than larger values. This phenomenon has been encountered previously and, again, is probably due to destruction of TPN (**6**). The values obtained with different incubation periods were not corrected to one time since the reaction in many cases

probably did not follow a linear course. All sources were tested with at least two different preparations and frequently with more. Considerable quantitative variation was observed among different preparations from the same plant tissue. Some sources gave extracts which became inactive on storage for two or three days; others were stable for months at $-15°$ C.

The data of table I and table II also show that, in addition to glutathione reductase, all but one of the various plant sources contained glucose-6-phosphate dehydrogenase activity. This fact is indicated by the data obtained when glucose-6-phosphate dehydrogenase was not added to the mixtures. Reduction of GSSG occurred in all cases except with extracts from sunflower leaves. In most instances, when the dehydrogenase was not added there was a smaller GSH titer, showing that the dehydrogenase,

TABLE III

DISTRIBUTION OF MALIC ENZYME IN PLANT TISSUES.

Tissue	Milliliters of 0.001 *N* $KIO_3 \cdot HIO_3$ (micromoles GSH formed)				
	Complete system	TPN omitted	GSSG omitted	Malate omitted	Mn^{++} omitted
Wheat germ	3.89	0.21	0.18	0.26	0.50
Etiolated pea seedling	1.62	0.05	0.08	0.12	0.67
Spinach leaf	2.10	0.18	0.20	0.20	0.26
Sunflower leaf	0.72	0.15	0.12	0.20	0.29
Sedum spectabile leaf	0.89	0.11	0.09	0.39	0.33
Parsley leaf and stem	2.25	0.11	0.08	0.11	0.15
Tomato leaf and stem	1.67	0.14	0.17	0.50	1.20
Cabbage leaf	2.48	0.15	0.12	1.04	1.40
Carrot root	4.66	0.09	0.12	0.24	0.50
Parsley root	3.15	0.09	0.08	0.14	0.27
Sweet potato root	0.54	0.12	0.14	0.14	0.18
Potato tuber	1.61	0.14	0.18	0.78	1.43
Avocado fruit	3.48	0.04	0.11	0.23	1.63
Cantaloupe fruit	3.11	0.42	0.15	1.01	2.25
Cucumber fruit	1.35	0.05	0.06	0.08	0.86

rather than the reductase was limiting the reaction rate. Omission of TPN, GSSG, or glucose-6-phosphate resulted in a considerable lowering of the titer. Blank titers in the absence of glucose-6-phosphate tended to be higher than the titers in the absences of other components and sometimes increased on storage of the frozen extract. Under such circumstances, a second dialysis lowered the blank titer, and it seems probable, therefore, that substrates for dehydrogenases requiring TPN are formed from high molecular weight compounds.

Malic enzyme and isocitric dehydrogenase

The various plant sources which had all been shown to contain glutathione reductase were also tested for malic enzyme and some sources were also tested for isocitric dehydrogenase. The results of tests for these two enzymes are reported in table III and table IV. The conditions employed

are those found to be optimal for the glutathione reductase-malic enzyme system in wheat germ extract. The complete system in table III is composed of GSSG, 1×10^{-3} M; TPN, 1×10^{-5} M; L-malate, 9.3×10^{-3} M; Mn^{++}, 1.1×10^{-3} M; collidine buffer, pH 6.8, 3.7×10^{-2} M; and plant enzyme preparation: three micromoles adenosine-5-phosphate added to the reaction mixture with cabbage leaf extract. The total volume was 2.7 ml. and the mixtures were incubated for 30 minutes at 30° C. The complete system in table IV is composed of GSSG, 1×10^{-3} M; TPN, 1×10^{-5} M; *dl*-isocitrate, 7.4×10^{-3} M; Mn^{++}, 1.1×10^{-3} M; collidine buffer, pH 6.8, 3.7×10^{-2} M; and plant enzyme preparation. The total volume was 2.7 ml., and the mixtures were incubated for 30 minutes at 30° C. All tests were positive except that for isocitric dehydrogenase in potato tuber. With cabbage leaf extract, adenylic acid was required to demonstrate the presence of malic enzyme. The interpretation of this phenomenon has been discussed in the previous

TABLE IV

DISTRIBUTION OF ISOCITRIC DEHYDROGENASE IN PLANT TISSUES.

Tissue	Milliliters of 0.001*N* $KIO_3 \cdot HIO_3$ (micromoles GSH formed)				
	Complete system	TPN omitted	GSSG omitted	Isocitrate omitted	Mn^{++} omitted
Wheat germ	4.91	0.27	0.18	0.27	3.51
Etiolated pea seedling	2.16	0.18	0.09	0.15	2.48
Spinach leaf	3.42	0.18	0.15	0.17	3.38
Sunflower leaf	0.84	0.14	0.14	0.18	0.78
Sedum leaf	0.44	0.11	0.09	0.15	0.20
Parsley leaf and stem	3.43	0.11	0.12	0.11	2.01
Parsley root	3.00	0.09	0.08	0.09	3.21
Sweet potato root	0.34	0.12	0.12	0.14	0.17
Potato tuber	0.96	0.11	0.20	0.83	0.45
Cucumber fruit	0.84	0.09	0.09	0.06	0.95

section. The blank values obtained in the absence of malate or isocitrate behaved similarly to the blank values without glucose-6-phosphate; this observation was also discussed in the previous section. Low blank titers were always obtained upon omission of GSSG or TPN.

Because of the known requirements of malic enzyme and isocitric dehydrogenase for Mn^{++}, this ion was routinely added to the test system. Omission of Mn^{++}, however, gave variable results. In the malic enzyme system, Mn^{++} always stimulated the reaction. Such stimulation was not always observed for the isocitric dehydrogenase system, and in a few cases addition of Mn^{++} resulted in a small inhibition. Failure to obtain activation with Mn^{++} may be ascribed in part to incomplete dialysis. This interpretation was shown to be true for the parsley leaf and stem preparation in which no stimulation by Mn^{++} of GSSG reduction by isocitrate was observed with an enzyme preparation which had been dialyzed for 24 hours. The same extract was then dialyzed for 48 additional hours, after which the results given in table IV were obtained. A definite stimulation by Mn^{++} is

apparent. The prolonged dialysis, however, had also resulted in a marked inactivation of the enzyme. The small inhibition caused by added Mn^{++} in a few cases might be ascribed to a requirement of TPN-destroying enzymes for Mn^{++}.

Discussion

Because of its simplicity, the method employed in this study is recommended as a test procedure for the presence of any TPN-linked dehydrogenase. It has been used as a quantitative assay procedure for glutathione reductase (**5**), and can probably be made quantitative for any component

TABLE V

SPECIFIC ACTIVITY OF ENZYMES IN PLANT TISSUES. THE ACTIVITY IS EXPRESSED AS MICROMOLES OF GSH PRODUCED PER GRAM DRY WEIGHT PER HOUR.

Family	Tissue	Glutathione reductase	Glucose-6-phosphate dehydrogenase	Malic enzyme	Isocitric dehydrogenase
Gramineae	Wheat germ	1460	1460	1140	1460
Leguminosae	Etiolated pea seedling	280	280	280	360
Chenopodiaceae	Spinach leaf	1380	540	800	1380
Compositae	Sunflower leaf	180	0	100	120
Crassulaceae	*Sedum spectabile* leaf	740	740	1440	860
Umbelliferae	Parsley leaf and stem	460	460	3000	1380
	Parsley root	1020	420	2280	2160
	Carrot root	340	40	580	....
Solanaceae	Potato tuber	54	42	1600	0
	Tomato leaf and stem	200	56	320	....
Cruciferae	Cabbage leaf	260	220	420	....
Convolvulaceae	Sweet potato root	38	26	56	28
Lauraceae	Avocado fruit	180	180	480	....
Curcurbitaceae	Cantaloupe fruit	1380	920	1080	....
	Cucumber fruit	5400	5400	3600	3000

of such coupled systems bringing about the reduction of GSSG, if the proper precautions and modifications are applied. Use of the malic enzyme–glutathione reductase system for the quantitative assay of small amounts of TPN will be described in another paper.

The results given in tables I, II, III and IV were obtained with extracts of tissues which varied widely in dry weight. In order to provide more nearly comparable figures for the enzyme activity of the proteins extracted from the various sources, the results have been calculated as micromoles GSH formed per gram dry weight per hour. These values have been summarized in table V. In each case, the GSH formed in the absence of added substrate was subtracted from the GSH formed in the presence of added

substrate. The first column gives the values obtained when GSSG was reduced by glucose-6-phosphate in the presence of excess glucose-6-phosphate dehydrogenase, and represents the glutathione reductase present. The other columns give the results obtained for GSSG reduction with each of the three dehydrogenase substrates, with no added enzyme. The figures of table V are quantitative only in the sense that they set a lower limit. The point to be stressed is not the magnitude of the numerical values, but rather, the uniformity of positive findings. Only two negative results were obtained (for glucose-6-phosphate dehydrogenase in sunflower and isocitric dehydrogenase in potato). It is possible that improved procedures would give positive findings in these instances.

The results show that glutathione reductase is present in a variety of tissues (roots, tubers, fruits, leaves and seeds) of eleven families of higher plants. The wide distribution in plants of glutathione reductase, TPN (**20**) and TPN dehydrogenases, accounts for the ability of plant tissues to reduce oxidized glutathione when it is added. RALL and LEHNINGER (**19**) have shown that glutathione reductase is also present in animal tissues. Oxidized glutathione is apparently reduced by similar mechanisms in most, if not all, living forms.

The question of the role of glutathione reductase in hydrogen transport has been discussed by MAPSON and GODDARD (**15**) and by CONN (**4**). Although there is more reason to assume such a function in the case of plant tissues than in the case of animal tissues, sufficient evidence is not yet on hand to warrant a conclusion that any appreciable fraction of respiratory hydrogen transport occurs by way of glutathione. The main function of the reductase may very well be the maintenance of glutathione in the reduced state.

Of the various dehydrogenase systems capable of reducing TPN, two of those studied in the present paper, malic enzyme and isocitric dehydrogenase, have already been shown to be widely distributed in plants (**1, 2, 6, 8, 21**). The substrates of these enzymes, malate and isocitrate, are also known to be common natural constituents of plant tissues. Furthermore, MAPSON and GODDARD (**15**) demonstrated, in the case of pea extracts, that malate and isocitrate were present in the extracts, and that the reactions shown in equations 4 and 5 actually took place.

In the case of glucose-6-phosphate dehydrogenase, however, little previous information was available on its occurrence in higher plants. In this connection, it should be pointed out that, since the reaction product or products of glucose-6-phosphate have not been identified in any of the experiments described here, there is a possibility that the reaction is more complex than the reaction shown in equation 2, *i.e.*, an oxidation of glucose-6-phosphate to phosphogluconic acid. In a number of instances, we have, in fact, found that the reducing action attributed in the text to glucose-6-phosphate dehydrogenase is due to the combined action of at least two dehydrogenases operating in sequence. Preliminary results obtained by Dr.

Robert C. Barnett and Dr. Helen A. Stafford with extracts from several plant sources indicate that glucose-6-phosphate is first oxidized to 6-phosphogluconate and that this compound is further oxidized by another TPN-linked dehydrogenase. Since the second reaction involves a liberation of CO_2, it is likely that the reaction sequence in higher plants is similar to the reaction sequence studied by HORECKER and his collaborators (**11**) in yeast, by COHEN and his collaborators (**3**) in bacteria, and by DICKENS and GLOCK (**7**) in animal tissue. The glucose-6-phosphate dehydrogenase activity demonstrated in the plant sources studied, may therefore be regarded as partial evidence that plant tissues are able to effect the oxidative breakdown of carbohydrate by way of phosphogluconic acid, and that ribose and other pentoses may be formed in this fashion.

Summary

A simple test has been described for determining the presence of glutathione reductase in dialyzed extracts of higher plants. Application of the test has shown that the enzyme is widely distributed in plant tissues. It was present in soluble form in all of the 15 different sources tested (wheat germ, pea seedlings, spinach leaf, sunflower leaf, *Sedum spectabile* leaf, parsley leaf, tomato leaf, carrot root, parsley root, sweet potato root, potato tuber, and the fruits of avocado, cantaloupe, and cucumber).

After glutathione reductase has been shown to be present in a particular extract, the presence of any other dehydrogenase requiring TPN in that extract may be determined readily by measurement of the reduction of oxidized glutathione in the presence of TPN and the substrate of the enzyme for which the test is conducted. Further observations on the widespread distribution of malic enzyme and isocitric dehydrogenase have been made with such coupled systems, using the sources listed above. In addition, such a test system has been used to show that the enzyme glucose-6-phosphate dehydrogenase is also widely distributed in the tissues of higher plants. The significance of these findings is discussed.

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EFFECT OF AGE OF LEAVES UPON THE RATE OF PHOTOSYNTHESIS IN SOME CONIFERS

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(WITH ONE FIGURE)

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Introduction

Information regarding the relationship between photosynthesis and the complex of internal factors encompassed by the term, age, may be of interest either from the point of view of basic science or of practical application. The literature on this subject appears to be rather meager. Singh and Lal (**2**) working with different aged leaves of flax, sugar cane, and wheat found that the rates of photosynthesis were slow for young leaves, increased to a maximum for mature leaves, and then decreased with increasing age. From studies on aging effects in Chlorella cultures, Winokur (**5**) reported that the rate of photosynthesis, expressed either per unit number of cells or per unit dry weight, increased during the first few days of a culture and then declined with increasing age of the culture. It would appear that those conifers which maintain their leaves for several years would be especially appropriate for the study of the effects of aging on processes in the leaves. Stålfelt (**3**) investigated the rates of photosynthesis in spruce and pine with respect to age of needles and light intensity. He concluded for spruce that the rate of photosynthesis, when calculated per gram of fresh weight of needles, appeared to decrease with increasing age of the needles only because the weight of the needles increased with age; whereas actually the rate per unit number of leaves increased with increasing age for five years before declining. The results were most marked at about 30% of full sunlight but were quite evident both below and above this light intensity. For pine, in 30% of full sunlight, he found a similar increase in the amount of photosynthesis per unit number of leaves with increasing age up to three years, the oldest used; but in full sunlight the rate was approximately the same for leaves of all ages.

From this review of literature, one may conclude that for some plants the peak photosynthetic capacity is reached at the time of maturity of the photosynthetic organ or structure, but in conifers the peak is not reached until the leaves are several years old. The purpose of the research reported in this paper was to obtain further data regarding the rates of photosynthesis in conifer leaves of varying age.

Methods and materials

The experimental plants used were Scotch pine, *Pinus sylvestris* L.; white pine, *Pinus strobus* L.; Austrian pine, *Pinus nigra* Arnold; western yellow

pine, *Pinus ponderosa* Laws.; white fir, *Abies concolor* Lindl. and Gord.; and Colorado blue spruce, *Picea pungens* Engelm. These plants were all young trees about fifteen feet tall, spaced widely as in specimen planting, and growing on or near the University campus. All experiments were performed from July through October after the leaves on the current year of growth were apparently mature. After a suitable branch was selected on a tree, it was cut into separate annual segments with the leaves intact. None of the trees had stem segments four or more years old with leaves in sufficient number or state of preservation for investigation; so leafy stems one (current), two, and three years old were employed in the study. In the laboratory, the basal end of each stem segment was cut off under water. The leafy stem sections were then placed in glass cylinders, 6 cm. × 30 cm., with the basal end of each stem in shallow water at the bottom of the container. Each cylinder contained leaves of a particular age. The cylinders were placed vertically in a constant temperature bath which was illuminated from one side by a combination of incandescent and fluorescent lights which gave a light intensity of 2200 foot-candles on the plants. In some experiments, especially with pines, the needles were removed from the more shaded portions of each stem to prevent excessive crowding and shading. The top of each cylinder was fitted with inlet and outlet tubes, the latter reaching nearly to the bottom, through which metered air was passed over the leaves at the rate of six liters per hour. The bath temperature was kept at 24° C.

Apparent photosynthesis was measured by determining the difference between the carbon dioxide content of the air before and after it passed over the plants. Carbon dioxide in the air was determined by the method described by WAUGH (**4**) which consists of measuring the changes in electrical resistance, at a constant temperature, of a dilute solution of KOH in a gas absorption bottle through which the air is passed. By repeated and simultaneous analysis of air from the same source with the four gas absorption towers used in this research, it was established that carbon dioxide in milliliters per liter of air at 24° C could be determined with a standard error of ± 0.007 which amounted to a maximum calculated discrepancy between the towers of less than 2%. At the close of each experiment, all leaves were removed from each annual stem segment for counting and volume and weight determinations.

Results and discussion

The common practice of expressing the rate of photosynthesis per unit area of leaf is not practical for the needle-shaped leaves of the gymnosperms used in this study. One is left with a choice between calculating the rate per unit volume, unit weight, or unit number of leaves, all of which can be easily and accurately determined. As noted earlier, Stålfelt concluded that both the fresh and dry weights of pine and spruce needles increased with age and that the rates of photosynthesis for leaves of different ages

expressed on this basis presented an erroneous picture, whereas calculation of the rates per unit number of leaves presented a different but true picture.

A preliminary survey of several conifers based upon samples of 400 to 500 leaves per year for leaves from one to four years old seemed to verify Stålfelt's conclusion that the fresh and dry weights of such leaves do increase with age. The volume was also found to increase in a similar manner. Occasionally, apparent exceptions were found but the over-all aspect was one of a measurable increase in weight and volume with an increase in age of the needles. Consequently, it was decided to calculate the rates of apparent photosynthesis simply on the basis of a unit number of leaves. It is from this point of view that all of the results pertaining to the rates of apparent photosynthesis with respect to age of leaves of various conifers are presented in tables I and II and figure 1.

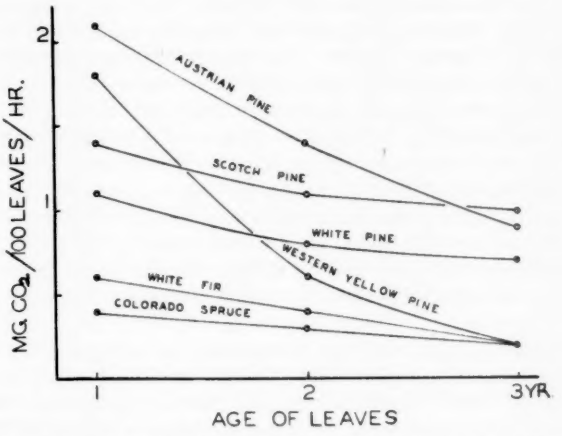

FIG. 1. Apparent photosynthesis in conifers with respect to age of leaves.

From these data, the leaves of all of the species investigated attained a rate of photosynthesis which was at or near the maximum by the time of apparent leaf maturity during the first season of growth. Since there are no data for the spring or early summer, it is not possible to establish precisely when the peak is reached. It is evident, however, that the photosynthetic capacity of the leaves decreases with increasing age beginning during the second year. These data are in accord with those published by SINGH and LAL (**2**) who reported that the maximum photosynthetic capacity was attained in some angiosperms at the time of apparent leaf maturity. The results do not confirm the conclusion of STÅLFELT (**3**) that the rate of photosynthesis increases with increasing age of needles up to three and five years for pine and spruce, respectively. Perhaps this discrepancy is more apparent than real since it does not appear that Stålfelt controlled temperature

TABLE I

APPARENT PHOTOSYNTHESIS IN CONIFER LEAVES WITH RESPECT TO AGE OF LEAVES. EACH VALUE IS AN AVERAGE OF TWO OR MORE MEASUREMENTS.

Plant	Number of trials	Photosynthesis in leaves		
		One year old	Two years old	Three years old
		mg. CO_2/100 leaves/hr.		
Colorado spruce	2	0.4	0.3	0.2
Austrian pine	4	2.1	1.4	0.9
White fir	4	0.6	0.4	0.2
Western yellow pine	2	1.8	0.6	0.2
White pine	5	1.1	0.8	0.7
Scotch pine	4	1.4	1.1	1.0

or made any correction for this factor whereas the data in this report were obtained under uniform conditions with respect to light, temperature, and concentration of carbon dioxide. The rates of photosynthesis of leaves of different ages for each species reported in the present paper were determined simultaneously which insured that in each experiment the previous environment was approximately the same for all of the leaves under consideration. Such uniformity was apparently not the case in Stålfelt's experiments. It should also be stated that Stålfelt noted some exceptions, especially in spruce, to the general conclusion that photosynthesis increases with increasing age of needles to five years. However, it is obvious both from the data in this report and those obtained by Stålfelt that the rate of photosynthesis in the leaves of certain gymnosperms may remain quite high for a period of two to three years.

In handling the needles of the various conifers throughout the course of the experiments, it was noted that the older ones particularly were often quite dark and dirty. It was thought that possibly the apparent differences between the results reported in this paper and those of Stålfelt might be due to the accumulation of soot and other dirt on the leaves. Several experiments were performed to test this hypothesis. The rates of photosynthesis

TABLE II

APPARENT PHOTOSYNTHESIS IN LEAVES OF CONIFERS BEFORE AND AFTER DIRT WAS REMOVED FROM THE LEAVES.

Plant	Condition of leaf surface	Photosynthesis in leaves		
		One year old	Two years old	Three years old
		mg. CO_2/100 leaves/hr.		
White fir	Uncleaned	0.7	0.5	0.3
	Cleaned	0.6	0.3	0.3
Spruce	Uncleaned	0.5	0.3	0.3
	Cleaned	0.6	0.4	0.3
Austrian pine	Uncleaned	1.3	0.9	0.8
	Cleaned	1.4	1.1	0.9

were first determined as usual; then the leafy branches were removed from the cylinders and the leaves were carefully, but thoroughly, scrubbed with a soft bristle brush and a dilute solution of either a detergent or soap. After scrubbing, the branches were rinsed in tap water and replaced in their respective containers for a second determination of photosynthesis. Since the results of all of these experiments follow the same trend, only representative samples are presented in table II. Examination of many of the leaves, both superficially and under a dissecting microscope, indicated that washing of the leaves was rather effective in removing the dirt. Nevertheless, the washing had no marked effects upon the order or magnitude of the rates of photosynthesis in the leaves of various ages. Therefore, it seems that the decrease in the rate of photosynthesis in conifer leaves, beginning during the second year, cannot be explained solely on the basis of the presence of dirt on the surface of the leaves at the moment. There is still a possibility that the accumulation of dirt on the leaves over an extended period of time may have induced secondary changes in the leaves which in turn may have resulted in a decrease in photosynthetic capacity. In view of the work by CLENDENNING and GORHAM (1) with respect to the effects of leaf ontogeny on the photochemical activity (Hill Reaction) of isolated chloroplasts, it seems likely that the decrease in photosynthesis with increasing age of leaves of conifers is due to internal cellular changes which occur normally with age. Clendenning and Gorham did not obtain active chloroplast suspensions from any of the four gymnosperms tried, but in using spinach and wheat they found that the photochemical activity of the chloroplasts increased as the leaves enlarged and matured and then decreased as the leaves became older. They reported that the order of activity of chloroplasts from young and mature spinach leaves was not altered by suspending the chloroplasts in the cell sap-cytoplasm from mature and young leaves, respectively, and therefore that changes in activity associated with age of leaf must be due to changes in the chloroplasts. If these conclusions should be found to hold true for gymnosperms, there would still be the problem of what change occurs in the chloroplasts which leads to an increase in photosynthetic capacity up to the maturity of the leaves, followed by a decrease in rate with further aging.

It is of interest, from the practical point of view, to know that the older leaves of conifers do have considerable photosynthetic capacity, even though it is less than in the leaves recently matured. Therefore, it seems safe to predict that pruning of conifers for shaping or other purposes will ordinarily not seriously impair the available supply of photosynthate in a branch or tree so long as no more than the youngest growth of the current season is removed.

Summary

The rates of apparent photosynthesis were determined for leaves of different age on the following conifers: *Pinus sylvestris* L., *Pinus strobus* L.,

Pinus nigra Arnold, *Pinus ponderosa* Laws., *Abies concolor* Lindl. and Gord., and *Picea pungens* Engelm. The leaves of all of the species investigated attained their maximum photosynthetic capacity approximately at the time of apparent leaf maturity during the first season of growth. Beginning during the second year of age, the rate of photosynthesis decreases slowly with increasing age of the leaves. The data indicate that the decrease in rate with increase in age is not due to the presence of dirt on the leaf surfaces. In spite of the decrease in rate with increasing age, conifer leaves three years old still have an appreciable photosynthetic capacity.

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THE RELATIVE RATES OF PHOTOSYNTHETIC ASSIMILATION OF ISOTOPIC FORMS OF CARBON DIOXIDE

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(WITH EIGHT FIGURES)

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Introduction

The dictum that isotopes of the same element are chemically the same is, of course, only an approximation. There are many examples of significantly different chemical behavior by different isotopes. Chemical reaction velocities often are quite unequal for different isotopes, particularly with the lighter (and biologically interesting) elements; and these differences may result in some degree of isotope separation. Certain chemical equilibria have been employed for the enrichment of some of the stable isotopes; isotope effects on reaction rate constants thus have been the basis for the production of tracers on a commercial scale. Therefore it is patently true that the use of tracers in biochemical studies may also involve chemical reactions at which some isotope separation occurs. In the case of a series of such reactions, it is easy to visualize an appropriate kinetic scheme whereby rather considerable isotope fractionation could result. As long as such fractionation is very small, it is of little direct concern to the tracer biochemist; if it should become very large, it could complicate and even invalidate his interpretation of tracer experiments.

Kamen (**5**) has called attention to this sort of complication in relation to tracer studies using the hydrogen isotope, H^3, or tritium. He pointed out that discrimination by chemical reactions between ordinary hydrogen, H^1 (protium), and tritium could, in theory, be so large as to completely invalidate the particular experiments considered.

More recently Bigeleisen (**2**) has called attention to the possibility that isotopic discrimination may complicate tracer studies even with elements heavier than hydrogen. His calculation of the minimal ratio of reaction velocity constants to be expected, for reactions involving C^{14} and C^{12} for example, was 0.67. Such a discrimination factor would be of real concern in tracer methodology; but it is actually a hypothetically extreme value arrived at by compounding second and higher order effects, all assumed to be maximally effective in the direction of enhancing rather than minimizing the discrimination. It seems very unlikely that isotope fractionation occurs to this maximal extent in a single actual reaction. It may be recalled, nevertheless, that the overall biological processes studied by physiologists or biochemists are rarely single reactions. Metabolic transformations in-

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volved in such processes as respiration, glycolysis, or photosynthesis are largely reversible reaction sequences in which single step fractionations conceivably may be additive leading to a much increased discrimination factor for the overall process. This situation would be analogous with the high efficiency with which a multiple-plate fractionating column effects the separation of liquids differing only slightly in their vapor pressures when a single distillation step achieves but little separation. Among the experimental studies demonstrating carbon isotope effects in relatively simple chemical reactions are those of BIGELEISEN and FRIEDMAN (**3**), STEVENS and ATTREE (**13, 14**), LINDSAY *et al.* (**6**), STEVENSON *et al.* (**16**), and YANKWITCH and CALVIN (**23**).

There is no lack of consensus regarding the fact of isotope discrimination by chemical or biochemical processes. The main concern here is the quantitative significance of such isotopic fractionation which has been the subject of considerable discussion most of which does not concern us directly. Only the discrimination between carbon isotopes which may occur in photosynthesis is considered. Some experiments have been reported which can be used to evaluate this effect.

NIER and GULBRANSON (**9**), NIER (**8**), MURPHY and NIER (**7**), BELKENGREN (**1**), and RABIDEAU (**10**) reported isotopic analyses of naturally occurring forms of carbon; and from these data on variation in isotopic ratios, it is apparent that the carbon of at least certain biological materials (*e.g.*, carbohydrates) characteristically contains less C^{13} than that of the atmospheric carbon dioxide from which it was synthesized. These data suggest indirectly that photosynthesis discriminates against C^{13} in that $C^{13}O_2$ is assimilated about 98% as fast as the normal $C^{12}O_2$.

Recently WEIGL and CALVIN (**18**) reported a much more extreme discrimination between $C^{12}O_2$ and $C^{14}O_2$ by photosynthesis in barley. WEIGL *et al.* (**17, 19**) supported this claim in more detail. The discrimination factor announced by Weigl was 0.83, *i.e.*, photosynthesis was found to proceed 17% slower with radioactive carbon dioxide. This factor was calculated from only one experiment although other experiments were said to agree qualitatively at least. These workers encountered numerous troubles with instrumentation which made quantitative evaluation of the other experiments not worth while. Weigl's experiment was the first actual kinetic experiment suggesting photosynthetic discrimination and had the apparent advantage of demonstrating the magnitude of discrimination from a direct comparison of photosynthetic reaction rate constants for $C^{12}O_2$ and for $C^{14}O_2$. In the same series of experiments, Weigl attempted to remeasure the C^{13}–C^{12} discrimination factor by the indirect method involving a comparison of the C^{13} content of algae with the C^{13} content of the carbon dioxide upon which the cells were grown. A discrimination of about 4% was computed from the analyses. This computation probably was subject to appreciable error since the conditions of measurement were not ideal and the experiment was not replicated.

It appears that an anomalous situation exists. Simple theory accounts for discrimination on the basis of the difference in mass between the isotopes being compared. For a reaction whose rate governing step is the opening of a bond between the isotopic atom and the adjacent atom in the molecule, it is reasonable to expect that the lighter the isotope, the lower the activation energy for the reaction; therefore the more rapid will be the reaction. One should predict, therefore, that the ratio of photosynthetic rate constants for $C^{12}O_2$ and $C^{13}O_2$ should be about the same as the ratio for $C^{13}O_2$ and $C^{14}O_2$. In other words, photosynthetic discrimination against C^{14} might be expected to be about twice as great as discrimination against C^{13} when the rate constants of these isotopic forms of carbon dioxide are compared with that of $C^{12}O_2$. From Weigl's results, it is learned that photosynthetic discrimination against $C^{14}O_2$ is from four to eight times the discrimination against $C^{13}O_2$, depending upon whether we accept for the C^{13}–C^{12} discrimination factor Weigl's value of 4% or a more nearly average value of 2%.

Both for theoretical interest and for practical reasons, it seemed desirable to reinvestigate this problem and to evaluate more carefully the relative photosynthetic velocity constants for the several isotopic forms of carbon dioxide. A series of kinetic studies were made which were similar in principle to those carried out by Weigl; but quite different and better methods were used for analysis of the isotopic forms of carbon dioxide. The results approximately confirm Weigl's findings. Unfortunately there are a number of objections to Weigl's methodology and some of these same intrinsic difficulties also prevent the results from being considered unequivocal. Therefore, the discrimination factors announced by Weigl and approximately confirmed here may be considered valid only on the basis of certain unproven assumptions. Existing data do not allow the testing of these assumptions.

Experimental methods

A recording mass spectrometer was employed to measure changes in the partial pressures of the three isotopic species, $C^{14}O_2$, $C^{13}O_2$, and $C^{12}O_2$, in the gas phase over the living cells. One instrumental method thus furnished all analyses. The mass spectrometer measurements were recorded on a strip chart. The accelerating voltage of the spectrometer was cycled automatically by a motor driven potentiometer so as to tune through the carbon dioxide masses 46, 45, and 44 once each cycle. A cycling rate of either 1.0 or 1.8 minutes per cycle was used. A section of a typical record is shown in figure 3 A. Thus, practically continuous records of masses 46, 45, and 44 were obtained throughout an experiment, and rates of change of the partial pressures of the isotopic forms of carbon dioxide to which they correspond could be compared. Technical details regarding the use of the mass spectrometer for a metabolic study of this kind have been presented previously (**4**).

In the present experiments, a gas circulating apparatus (fig. 1) somewhat different from the system described previously was employed. The

glass system shown in figure 1 had a volume of about 50 ml. The circulating pump was operated at a pumping speed of 180 ml. per minute providing a complete turnover of the gas within the system every 17 seconds. A very small leak through which the system communicated via a T-connection to the mass spectrometer provided for continuous sampling of the gas within the system. The rate of gas flow through the leak was of the order of a few milliliters per day.

The mass spectrometer leak, located as near as possible to the T-connection with the gas circulating system, was constructed as previously described (**4**). Since it was desirable to measure accurately very low partial pressures of carbon dioxide, it was necessary to use a rather higher rate of gas

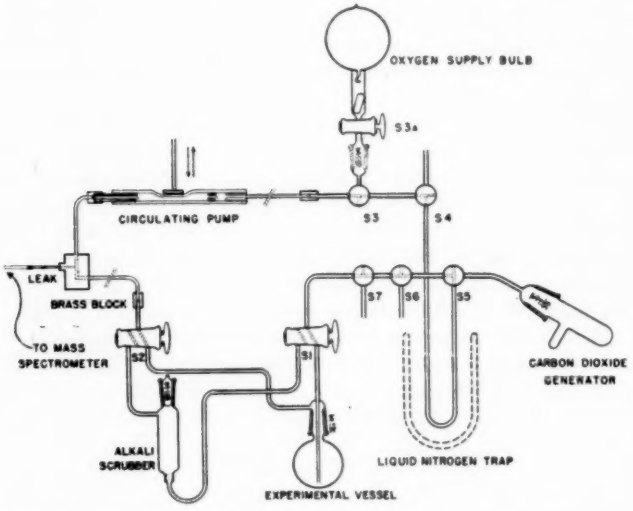

FIG. 1. Diagram of apparatus. The functions of the system components are described in the text.

flow through the leak than is the usual practice in mass spectrometry. The pressure in the spectrometer tube therefore was considerably higher than would be considered optimal and special care was taken to keep the partial pressure of oxygen within the system well below 100 mm. Hg in order to protect the tungsten filament in the spectrometer. Most of the data were obtained in an atmosphere containing about 1 to 2% oxygen.

It was essential that the apparatus be closed to the surrounding atmosphere. The system was tested for leaks by evacuating with an oil diffusion pump. Ability to attain a minimal pressure, read on a thermocouple gauge, indicated the absence of all but the very smallest leaks. Further testing was done by the mass spectrometer itself. The instrument was tuned to mass 32 (oxygen); and the apparatus either was filled with an inert gas of

low oxygen content or, in a more rigorous test, was evacuated. An increase in mass 32 was the most sensitive device for detecting air leaking into the apparatus.

Stopcocks present in the system allowed for the introduction of appropriate gas mixtures. As a typical example, water-washed helium or nitrogen was introduced at S_7 (fig. 1) and flushed through the system to the atmosphere at S_6. Reversal of S_6 and S_7 then flushed the short section between these stopcocks. When required, oxygen was introduced either as air or from an oxygen supply bulb as shown in figure 1. The section from S_{3A} was evacuated through S_4, gas from the bulb was allowed to diffuse into the section between S_3 and S_4; and when the circulating pump was turned on, this oxygen was completely mixed throughout the system in three to four minutes.

Isotopically enriched carbon was available as barium carbonate which could have been converted to enriched carbon dioxide gas and delivered to the system from a supply bulb in the same manner as the oxygen. It was easier, however, to control the exact quantity of the tracer to be delivered by weighing out the desired amount of carbonate and quantitatively converting it to carbon dioxide within the apparatus by means of the carbon dioxide generator shown in figure 1. Solid barium carbonate was placed in the bottom of the generator flask. An excess of sulphuric acid was added carefully to the side arm. The generator was assembled and the acid was frozen by surrounding the generator with a dry ice bath. The section between S_4 and S_6 including the generator was evacuated. After allowing the acid to thaw, the generator was rotated around its ground joint tipping the acid onto the carbonate and generating isotopically enriched carbon dioxide. The gas was frozen out in the trap surrounded by a liquid nitrogen bath to insure complete transfer from the generator flask. Stopcocks were turned to shut off the generator flask at S_5 and to include only the circulating system proper. The trap was permitted to thaw and the system was brought to a pressure of one atmosphere with helium. In this manner about 0.5 ml. of carbon dioxide was introduced bringing the content of this gas in the system to approximately 1% at the beginning of an experiment.

An absolute calibration of the carbon dioxide concentration measurements was not required, but it was essential that the relative partial pressures of the different isotopic forms of carbon dioxide be known accurately. As enriched carbon dioxide disappeared from the gas phase the spectrometer records of masses 46, 45, and 44 all reached low limiting values significantly above the amplifier zero. The true relative partial pressures of the corresponding $C^{14}O_2$, $C^{13}O_2$, and $C^{12}O_2$ could only be evaluated if their respective mass spectrometer readings for zero partial pressure of carbon dioxide could be obtained. The usual spectrometer residual values proved unreliable as base line corrections. It was necessary to obtain valid base lines for each form of carbon dioxide by removing the gas from the circulation stream. The removal was accomplished at an appropriate time during or at

the end of an experiment by turning stopcocks, S_1 and S_2 of figure 1, and routing the gas stream through the scrubber containing 5% sodium hydroxide. The steady values for masses 46, 45, and 44 which were obtained thereafter were taken as base lines corresponding to an absence of carbon dioxide.

With this procedure, the precision of isotope analyses requisite for the experiments was obtained as could be demonstrated by a comparison of the rates of uptake of two isotopic forms of carbon dioxide in 5% KOH. For this test, a drop of alkali was deposited on filter paper which in turn was placed within a small bakelite cover so as to reduce its effectiveness as a carbon dioxide absorbant. With such a relatively inefficient absorbing system, about 45 minutes were required for complete disappearance of a sample of isotopically enriched carbon dioxide from the gas phase within the experimental chamber employed. Figure 2 A shows the time course of the absorp-

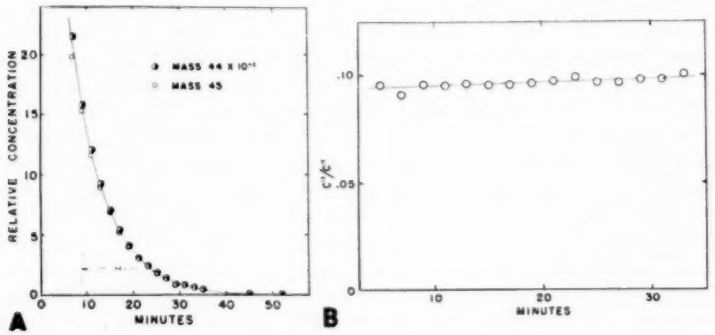

FIG. 2. A. Time course of solution of isotopically enriched carbon dioxide in alkali. Ordinate values are relative partial pressures of $C^{12}O_2$ and $C^{13}O_2$ remaining in the gas phase. B. Isotope ratios, mass 45/mass 44, calculated from the data of A.

tion in this control experiment. The only difference to be expected in the rates at which the two isotopic forms of carbon dioxide disappeared would be that due to different velocities of diffusion. Since the calculated difference is only about 1%, such a diffusion difference could not be measured accurately. When the isotope ratio, mass 45/mass 44, is computed from these data, it is evident (figure 2 B) that the ratio does not change significantly indicating the absence of isotope discrimination. The very slight deviation from zero slope in figure 2 B happens to be exactly the amount predicted on the basis of diffusion rate difference. No doubt this agreement is fortuitous. Results such as these could not be obtained if there existed a large systematic error in the overall method.

The experimental vessel had a useful liquid volume of 10 ml. When the vessel contained a suspension of algae, the gas stream bubbling through the suspension at about 3 ml. per second provided stirring which was more than adequate. Foaming proved troublesome only occasionally.

A 200 watt tungsten filament projection lamp was used in a housing fitted with appropriate condenser lenses to give a light beam larger than the experimental vessel. The light passed through 5 cm. of saturated $FeSO_4$ in 25% H_2SO_4. With this filter in place, no significant temperature rise occurred in the experimental vessel. The light intensity was somewhat above 1000 fc. The exact value was not accurately measured. The light incident upon the experimental vessel therefore was white in color, it illuminated the entire vessel, and it was of an intensity corresponding approximately to photosynthetic saturation for the green algal suspensions used. With this type of illumination on the suspension stirred by the circulating gas stream, all algae cells received equal exposure to light. The C^{14} was obtained from the U. S. Atomic Energy Commission as 6% enriched barium carbonate. The C^{13} was obtained from two sources. Barium carbonate, of which 10% of the carbon was C^{13}, was prepared by Dr. A. O. C. Nier. Barium carbonate, of which 61.5% of the carbon consisted of C^{13}, was purchased from Distillation Products Industries.

The organism used in most experiments was *Chlorella pyrenoidosa* Chick. The culture was obtained originally from Dr. Robert Emerson and has been maintained by various individuals for several years. Cultures were grown autotrophically in continuous fluorescent light in a modified Knop's solution. Air containing 4% carbon dioxide was bubbled through the cultures which were maintained at 27° C in an air-conditioned cabinet during four to six days prior to harvesting the cells. The culture suspensions were harvested by centrifuging. The cells were washed once with water and resuspended to a density of 1% by volume in 0.1 M phosphate buffer, pH 5.4, in which the ratio of K/Na was 12.5.

In some experiments barley leaves were used. Experimental material was obtained from seedlings of the Velvet variety which were grown in the greenhouse for two to three weeks. Washed segments of leaves were placed against the inner face of the experimental vessel with a forceps and teased into position so that they did not overlap. The leaf blades were then normal to the direction of incident light and presented a maximal area for illumination. Liquid medium was not used in such experiments.

Results and discussion

To interpret the data from an experiment, it was necessary to measure the height of each recorded mass peak (fig. 3 A). Values were corrected for recorded sensitivities which differed for different mass peaks. Then, to facilitate comparison, measurements within each cycle were made to coincide in time by interpolation on the time axis. One additional correction was required because of the contribution of oxygen isotopes to masses 45 and 46. Isotopic forms of carbon dioxide contribute to all masses from mass 44 through mass 50, and each mass above 44 consists of a family of isotopic carbon dioxide molecules. Mass 45, for example, is composed of $C^{12}O^{16}O^{17}$ and $C^{13}O^{16}O^{16}$. Mass 46 is made up of four constituents:

$C^{14}O^{16}O^{16}$, $C^{12}O^{16}O^{18}$, $C^{13}O^{16}O^{17}$, and $C^{12}O^{17}O^{17}$. The probability of existence of a molecule containing two of the least common isotopes is small and their contribution to the total carbon dioxide could be ignored. However the relative accuracy required by the experiments necessitated correcting each mass peak for contributions by the oxygen isotopes. These corrections were based on the consideration that any photosynthetic discrimination which

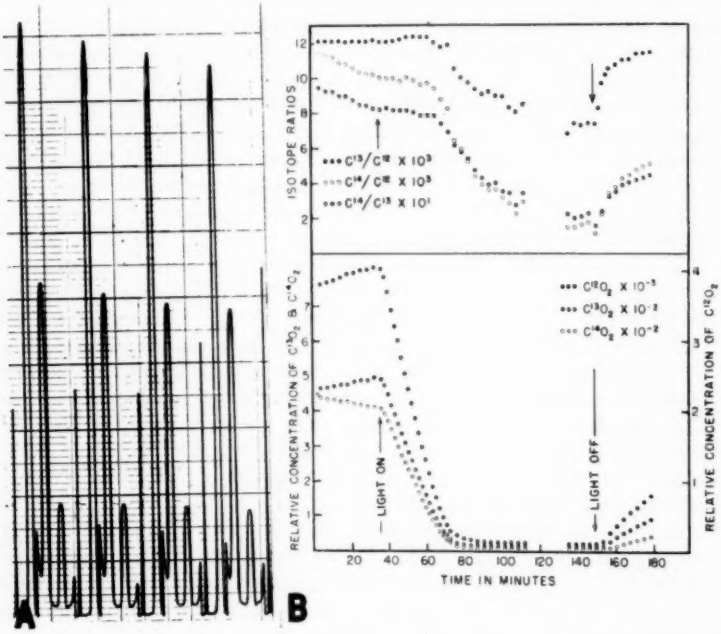

FIG. 3. A. Photograph of sample strip chart data. Four complete tuning cycles are shown. Peaks reappearing in successive cycles correspond to masses 44, 45, and 46. Tallest set of peaks are mass 44 (recorded at 1/100.1 maximal sensitivity). Middle set of peaks are mass 45 (recorded at 1/5.06 maximal sensitivity). Lower set of peaks are mass 46 (also recorded at 1/5.06 maximal sensitivity). Abrupt baseline shifts between 44 and 45 peaks are due to automatic changes of amplifier sensitivity. The sharp spike at the end of each cycle is the back trace as the accelerating voltage is rapidly returned to the starting point for the next cycle. B. Record of a complete Chlorella experiment. Lower graph shows progressive change of each isotope; isotope ratios are plotted above.

might be found should relate to the carbon isotope rather than to the mass of the whole carbon dioxide molecule. According to NIER (8), the natural abundances of oxygen isotopes are O^{16}, 99.759%; O^{17}, 0.0374%; O^{18}, 0.2039%. Assuming that oxygen isotopes are present in their natural abundance ratios in carbon dioxide, the fraction of each carbon isotope present can be computed from mass ratio data as follows. $C^{12}O_2$ accounts for all of mass 44,

plus that portion of mass 45 in which either of the two oxygen atoms is O^{17}, and that portion of mass 46 in which either of the two oxygen atoms is O^{18}. By extending these considerations to include $C^{13}O_2$ and $C^{14}O_2$, the following formulae are derived in which (44) refers to the partial pressure of mass 44, *etc.*:

$$\frac{C^{12}O_2}{CO_2} = \frac{(44)}{(44)+(45)+(46)} + \frac{2 \cdot 0.000347 \cdot C^{13}O_2}{CO_2} + \frac{2 \cdot 0.002039 \cdot C^{12}O_2}{CO_2}$$

$$= 1.00485 \frac{(44)}{(44)+(45)+(46)}$$

$$\frac{C^{13}O_2}{CO_2} = \frac{1.00485\,(45) - 0.0007553\,(44)}{(44)+(45)+(46)}$$

$$\frac{C^{14}O_2}{CO_2} = \frac{1.00485\,(46) - 0.0007553\,(45) - 0.004117\,(44)}{(44)+(45)+(46)}$$

After correction, the measurements had the dimensions of relative atomic concentrations of the respective carbon isotopes.

It is apparent that the relative C^{14} concentration, which corresponded to the corrected value for mass 46, was subject to the greatest error since its computation depended on measurements of masses 44 and 45 as well as of mass 46 itself. Also errors were largest at very low 46/44 mass ratios where corrections for isotopic oxygen were proportionately larger. Nevertheless the precision of the measurements was adequate for the purposes of the experiments. Corrected values computed from strip chart data in a representative experiment were plotted in the lower part of figure 3 B. This graph shows the time course of carbon dioxide metabolism by a Chlorella suspension in dark and light. Initially the gas phase contained about 2% carbon dioxide slightly enriched above the natural abundance of C^{13} and having a C^{14} content about equal to that of C^{13}.

In the initial dark period, C^{12} and C^{13} increased due to respiration by the algal cells. The decline in C^{14} is evidence that isotope exchange was occurring with the result that the carbon of the gaseous carbon dioxide was becoming equilibrated with exchangeable carbon atoms of cellular constituents of the algae. Upon illumination, all three carbon isotopes were assimilated until low steady values were attained at which time production of each isotope exactly balanced consumption (carbon dioxide compensation). After passing the gas stream through the alkali scrubber to determine base lines for this experiment, the vessel was made dark and the carbon dioxide which was subsequently evolved contained all three carbon isotopes as illustrated.

It would be convenient if relative photosynthetic velocity constants could be computed directly from such data. Any differences in photosyn-

thetic rate constants for the several carbon isotopes must tend to alter the isotope ratios; and in order to show these changes graphically, isotope ratios were computed for the data of figure 3 B and were plotted above on the same time scale. A serious complication became apparent. In the initial dark period, dilution of the C^{14} in the gas phase proceeded at a relatively high rate. The ratios, C^{14}/C^{12} and C^{14}/C^{13}, declined rapidly; and if photosynthetic discrimination occurred after the light was turned on, this should have led to an increase in these ratios as would have been evidenced by a positive change in the slope of the ratio curves. However a decrease either in the rate of respiration or of exchange during illumination would have also changed the slope of the ratio curves in the same way. It is impossible to

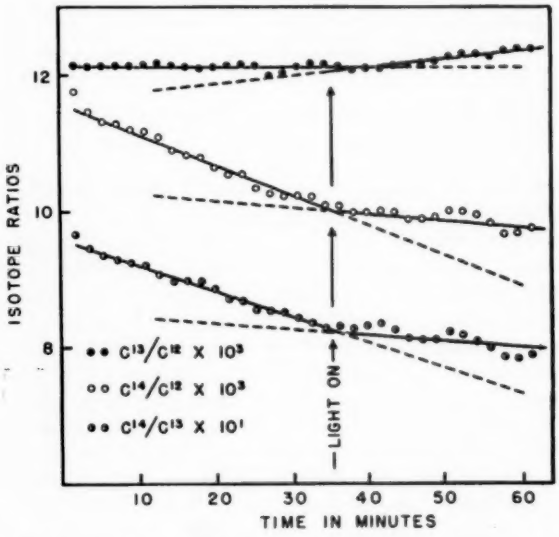

FIG. 4. Enlargement of a portion of figure 3 B to show changes in the slopes of isotope ratio curves at the beginning of illumination.

decide from these data whether respiration and exchange continued unabated during the light. A positive increment in the slope of the isotope ratio curves which actually occurred at the time the light was turned on is shown in figure 4 which is an enlargement of the relevant portion of the isotope-ratio graph of figure 3 B. Straight lines were drawn by the least squares method through the points of each curve intersecting at the beginning of illumination. Similar data were obtained in other experiments of which figures 5 and 6 are representative.

If it is assumed that illumination affects neither the isotope exchange rate nor the rate of respiratory production of carbon dioxide, then it is apparent that the changes in the slopes of isotope ratio curves can be used

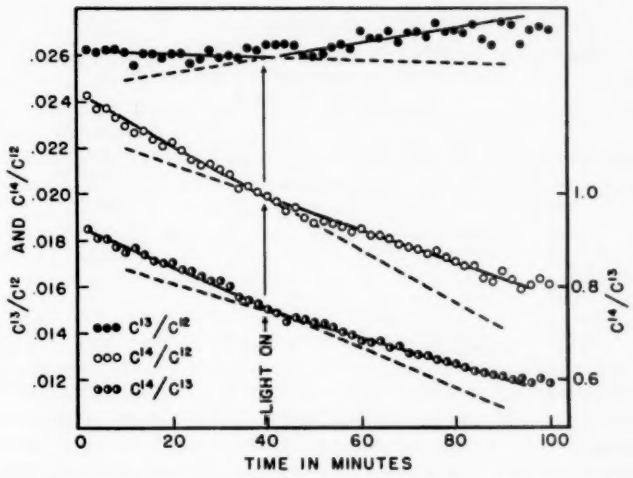

FIG. 5. Data similar to those shown in figure 4 from another Chlorella experiment.

to evaluate the relative photosynthetic velocity constants for the different carbon isotopes. Calculations were performed in the following manner. In each experiment, after a brief induction period at the beginning of illumination, the rate of assimilation of carbon dioxide remained constant until photosynthesis became inhibited by low pCO_2. For each of 10 to 20 con-

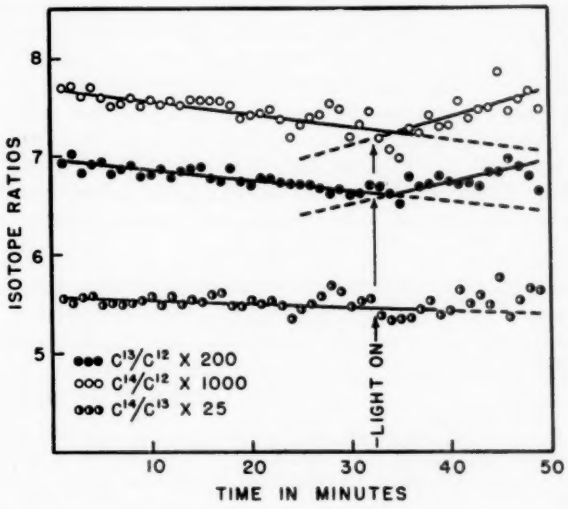

FIG. 6. Data similar to those shown in figures 4 and 5 from a barley experiment.

TABLE I

SAMPLE CALCULATIONS OF RELATIVE PHOTOSYNTHETIC VELOCITY CONSTANTS FROM RATES OF UPTAKE OF THREE CARBON ISOTOPES.

Isotope	Time	Total isotope (C)	Increment (ΔC)	ΔC after dark correction	C interpolated to midpoint of time interval	$\frac{\Delta C}{C} = X$
	min.					
C^{12}	48	23191				
	49	21031	2160	2309	22111	0.1044
C^{13}	48	285				
	49	259	26	28	272	0.1029
C^{14}	48	234				
	49	212	22	20	223	0.897

secutive intervals during this period the ratio, $\Delta C/C$ was computed for each isotope. Since ΔC was assumed to be due partly to dark respiration and exchange, each increment was corrected by an amount corresponding to the rate of change of that isotope in the preceding dark period. Each corrected value of ΔC was divided by the average concentration of the isotope during the interval represented and the resulting quotient, X, was a measure of the photosynthetic rate constant for that isotope. Ratios of X values are thus ratios of rate constants. Table I shows sample calculations for one time interval of one experiment. The ratio of X for C^{14}/X for C^{12} is 0.86 and X for C^{13}/X for C^{12} is 0.99.

Table II shows averages of such ratios of rate constants from four separate experiments. In the absence of photosynthetic discrimination, these ratios all would be unity. The results indicate that photosynthetic discrimination against C^{14} occurs to the extent of 15% and against C^{13} to the extent of 4%. These values agree approximately with those reported by Weigl and coworkers which they calculated from their best experiment.

It is regretted that these results, to some extent at least, must be considered equivocal and that the agreement with Weigl's findings is only superficial. These calculations are based on the assumption that both respiratory production of carbon dioxide and isotope exchange rates are

TABLE II

RELATIVE VELOCITY CONSTANTS AND THEIR STANDARD ERRORS FOR PHOTOSYNTHETIC UTILIZATION OF ISOTOPIC FORMS OF CARBON DIOXIDE.

Experiment number	Plant	$\frac{X^{13}}{X^{12}}$	$\frac{X^{14}}{X^{12}}$	$\frac{X^{14}}{X^{13}}$
0103	Barley	0.98 ± .06	0.88 ± .07	0.89 ± .09
1503	Chlorella	0.94 ± .04	0.80 ± .05	0.86 ± .06
0504	Chlorella	0.98 ± .03	0.87 ± .03	0.88 ± .04
2607	Chlorella	0.95 ± .04	0.86 ± .03	0.91 ± .04
Averages		0.96	0.85	0.89

unaffected by light. Weigl and coworkers did not make this assumption but were able to compute approximately the same discrimination factors only if they assumed a 50% photoinhibition of cellular production of carbon dioxide, and they ignored the effects of exchange which was justified incorrectly by defining all production of carbon dioxide as respiration and all uptake as photosynthesis. However, if isotope exchange rates are high, then a significant fraction of the cellular incorporation of tracer carbon is not truly photochemical. Therefore much significance cannot be attached to the agreement between Weigl's final result and this one.

Solubilities of isotopic species of carbon dioxide

Implicit in this interpretation of the data presented is the assumption that the different isotopic forms of carbon dioxide are equally soluble in the liquid phase which is present. This same assumption also must apply to the data of Weigl. Unfortunately neither experimental evidence nor adequate theory by which this assumption may be tested is known. The complication arising from a possible difference in solubilities of different kinds of carbon dioxide is readily apparent from a consideration of the expression for the isotope discrimination factor or, as used above (table I), the ratio of relative photosynthetic velocity constants. This ratio was computed for the case of $C^{14}O_2$ compared with $C^{12}O_2$, as

$$\frac{\Delta C^{14}}{C^{14}} \Big/ \frac{\Delta C^{12}}{C^{12}}$$

where ΔC^{14} and ΔC^{12} refer to observed increments of mass 46 and mass 44 (corrected for oxygen isotopes) and C^{14} and C^{12} refer to the average partial pressures of these molecular species prevailing during the time interval considered. A more rigorously derived expression for this ratio of rate constants is

$$\left(\frac{dpC^{14}O_2/dt}{dpC^{12}O_2/dt}\right)\left(\frac{G + \alpha_{14}\,L}{G + \alpha_{12}\,L}\right)\left(\frac{pC^{12}O_2\,\alpha_{12}}{pC^{14}O_2\,\alpha_{14}}\right)$$

in which $pC^{14}O_2$ and $pC^{12}O_2$ refer to partial pressures of the two forms of carbon dioxide in the gas phase, α_{14} and α_{12} are the aqueous solubility coefficients for the respective gases, G is the gas volume, and L is the liquid volume of the experimental system. The second and third terms in this expression cannot be evaluated without knowledge of α_{14}. Upon the assumption that $\alpha_{14} = \alpha_{12}$ the expression reduces to a useful form which can be evaluated from our experimental data, *viz.*

$$\left(\frac{dpC^{14}O_2/dt}{dpC^{12}O_2/dt}\right)\left(\frac{pC^{12}O_2}{pC^{14}O_2}\right)$$

In the absence of this assumption, the same simple expression is approximated only if G becomes very small compared with L. Thus, in an experimental system without any gas space, the value of a_{14} is of no concern in the computation of photosynthetic velocity constant ratios. It is technically infeasible to design an experimental chamber without any gas space though G could be made negligible. This was not the case with our apparatus for which G/L was about 4 or greater; nor was that condition fulfilled in Weigl's apparatus in which G/L was very large. In view of the importance of this matter, it is hoped that the aqueous solubility of $C^{14}O_2$ will some day be determined experimentally.

Absence of respiratory photoinhibition

An attempt has been made to test the assumption that, in these experiments, respiration was unaffected by light. For this purpose, oxygen exchange by the plant material has been measured since isotope exchange with molecular oxygen does not occur under the experimental conditions. Upon the introduction of oxygen highly enriched in O^{18} into the gas phase of the apparatus, the oxygen consumed by respiration was tagged whereas that produced by photosynthesis from water was of normal isotope composition. The tracer oxygen was present as $O^{16}O^{18}$ (mass 34) and was used at an isotope ratio, 34/32, of about 0.4. This ratio for naturally occurring oxygen is 0.004 so that enrichment was 100-fold. All other conditions were essentially the same as in previous experiments. If light should completely inhibit respiration, tracer oxygen would be consumed by the green plant cells under observation only in the dark, and no decline in partial pressure of oxygen of mass 34 would be observed in the light. On the other hand, if no photoinhibition of respiration occurred, practically the same rate of decrease of mass 34 dark or light should be expected. Figure 7 illustrates such experiments for Chlorella and barley. There appears to be no justification for ascribing any major inhibitory effect on the rate of tracer oxygen consumption to illumination. These results are representative of data from a number of similar experiments under conditions identical with those which governed the studies on isotopic carbon dioxide exchange. As far as oxygen is concerned, significant photoinhibition of respiration may be ruled out. Perhaps the same is true for carbon dioxide production but one cannot be so certain in that case.

The kinetic separation of simultaneously occurring respiratory consumption and photosynthetic production of oxygen is of general interest particularly in relation to the validity of the usual dark correction in measurements of the true photosynthetic rate. More extensive measurements of this kind will be reported in a subsequent paper.

Isotope exchange between carbon dioxide and cellular carbon

Isotope exchange reactions involving carbon dioxide have frequently been demonstrated in a variety of organisms in both plant and animal kingdoms since the classic observations of Wood and Werkman (**20, 21, 22**).

Dark uptake of tracer carbon dioxide by exchange reactions in Chlorella and barley was first noted by RUBEN *et al.* (**11, 12**) with $C^{11}O_2$. In these experiments, it was apparent from the fall in $C^{14}O_2$ in the dark that $C^{12}O_2$ and $C^{13}O_2$ must be produced by exchange in excess of the net production of these isotopes by respiration. Weigl, Warrington, and Calvin, while certainly aware of such exchange reactions, did not consider them as such nor do their data show any indication of isotope exchange at a significant rate. No satisfactory explanation for this discrepancy between their data and these can be offered.

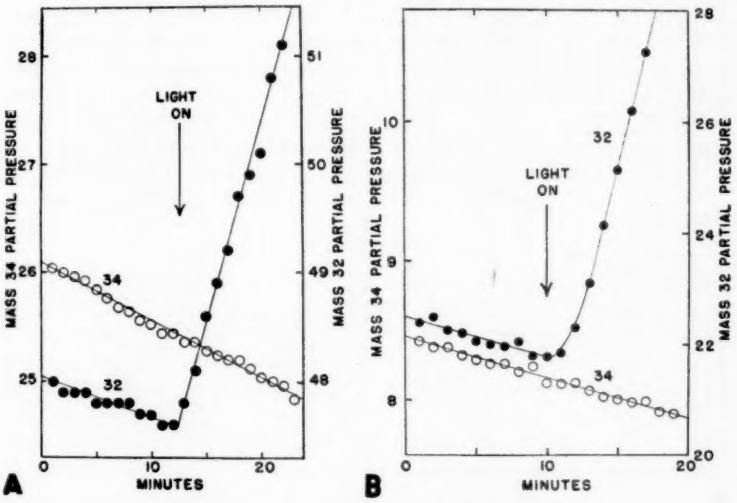

FIG. 7. A. Time course of partial pressures of oxygen isotopes during dark and light intervals of an experiment with Chlorella. The curve for mass 32 shows oxygen consumption by the cell suspension in the dark, oxygen production in the light. The curve for mass 34 shows only respiratory depletion of the tracer proceeding at essentially the same rate light or dark. Since no significant quantity of mass 34 is produced during photosynthesis, oxygen production by that process does not interfere with the determination of respiration rate during illumination. B. Data from an experiment on barley comparable with the one illustrated in A.

A priori one cannot be certain that carbon isotope exchange will be sufficiently rapid to be of significance for the calculation of photosynthetic velocity constants. If the magnitude of this isotope exchange was small, photoinduced changes might be negligible; however this was not the case with this material, as was found by direct measurement of the exchange rate, using for convenience carbon dioxide with high C^{13} enrichment. Chlorella cells and barley leaf tissue were observed in the dark with no carbon dioxide initially added to the gas phase. Rates of respiratory production of C^{12} and C^{13} were determined. Then the gas phase was enriched with tagged

carbon dioxide containing 61.5% C^{13} and isotope exchange was observed as shown in figure 8 for Chlorella and barley, respectively.

It should be noted that the addition of tracer carbon dioxide did not alter the total rate of production of this gas in either experiment and that the concentration of tracer carbon dioxide fell very rapidly as soon as measurements were resumed. It is apparent that production of $C^{12}O_2$ by the cells was greatly accelerated in exchange for absorption of $C^{13}O_2$. The rate of this exchange reaction can be estimated from these data. For the two experiments illustrated by figure 8, the exchange rates (computed on a one-

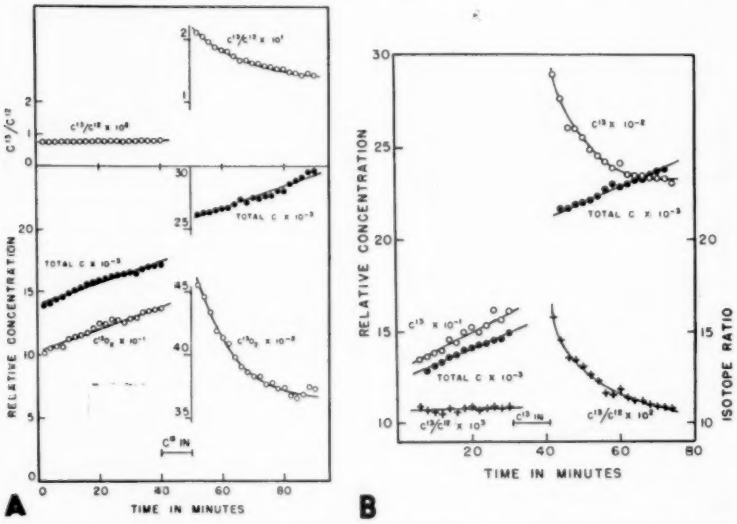

FIG. 8. A. Dark metabolism of isotopically enriched carbon dioxide by a Chlorella suspension. Data below show relative partial pressures of $C^{13}O_2$ and of total CO_2 increasing due to respiratory production. Between 40 and 50 minutes, C^{13} enriched carbon dioxide was introduced. Rate of total respiratory carbon dioxide production was unchanged. Rapid isotope exchange is demonstrated by accelerated production of $C^{12}O_2$ balanced by incorporation of $C^{13}O_2$. Upper graph shows time course of isotope ratio: constant initially, rapidly changing after isotope enrichment. B. Data from an experiment with barley similar to that illustrated by A.

way basis) were three and one half and four and one half times the respiratory rates. Therefore, isotope exchange certainly was a significant factor in the experiments, and no way can be seen to confirm unequivocally the assumption that the exchange rates are not photosensitive. To this extent the conclusions presented here are tentative.

DISPROPORTIONATE DISCRIMINATION AGAINST C^{14}

In the experiments reported here, the relative photosynthetic velocities for the several isotopic forms of carbon dioxide were consistently in ratios

which indicated from three to six times as great an isotope effect for C^{14} as for C^{13}. The data in table II indicate what significance may be attached to the average value of this disproportionate discrimination. For a single reaction, the predicted C^{14} effect should be only about twice the C^{13} effect. For a series of reversible sequential reactions, the C^{14} effect in the overall process can become greater than twice the C^{13} effect as the fractionations are compounded at each reaction step. One can easily show, however, that even a threefold difference in C^{14} and C^{13} isotope effects could be brought about in this manner only if an impossible large number of fractionation steps is assumed or else with much larger single step isotope effects than are indicated by the overall result. No physical theory which can account satisfactorily for the significantly greater effect of C^{14} is known. This anomaly, which was observed in each experiment, is not attributed to some systematic error of unknown origin. Also it may be pointed out that these observations are not unique; recently a similar anomaly was reported (**15**) for a chemical reaction in a non-biological system where the C^{14} effect was 2.7 times the C^{13} effect. At present, such experimental findings may be considered a challenge to the theoretician.

Summary

Metabolic production and absorption of isotopic forms of carbon dioxide were measured with a recording mass spectrometer. *Chlorella pyrenoidosa* suspensions and barley leaf tissue were studied. Calculations based upon changes which occurred in the slopes of isotope ratio (C^{13}/C^{12}, C^{14}/C^{12}, and C^{14}/C^{13}) curves upon illumination indicate an isotope discrimination by photosynthesis. $C^{12}O_2$, $C^{13}O_2$, and $C^{14}O_2$ are utilized at rates which apparently are in the ratios of 1.00 : 0.96 : 0.85 respectively. Computation of these ratios was based upon the assumption that light does not affect metabolic processes involving production or consumption of carbon dioxide other than photosynthesis. This assumption applies to carbon dioxide production by respiration and to isotope exchange which involves uptake of tracer carbon dioxide without altering the net rate of gas exchange. In the case of respiration, light was shown not to affect significantly the rate of oxygen consumption under the experimental conditions employed as was demonstrated by tagging the respiratory oxygen. In the case of isotope exchange, the rate was measured and found to be several times the rate of net production of respiratory carbon dioxide. Evidence at hand does not permit a decision as to the photosensitivity of the exchange rate.

The isotopically enriched oxygen and some of the C^{13} enriched carbon used in these studies were prepared by Professor Nier under a grant from the American Cancer Society through the Committee on Growth of the National Research Council. The authors wish to acknowledge the support given while the senior author was an Atomic Energy Commission Predoctoral Fellow in the Biological Sciences.

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TEMPERATURE OF POTATO AND TOMATO LEAVES[1]

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(WITH ONE FIGURE)

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Introduction

The energy economy of leaves is of fundamental importance because leaves are one of the major sites where solar energy is utilized in the synthesis of food. The energy economy is of further importance because it determines the temperature difference between leaf and environment. This difference influences water relations and the growth of parasites. Earlier proposals (**3, 4, 15**) for an energy economy are integrated in the present paper. The usefulness of the resulting economy was tested in the field using the proposed economy to predict the temperature differences between leaf and environment and then comparing these predictions with observations in the field.

Materials

During July, 1950, thermoelectric measurements of plant part temperatures were taken in potato and tomato plots at Clear Lake, Iowa, and Wanatah, Indiana. In August, 1951, additional measurements were taken in tomato plots at Ames, Iowa. At Clear Lake, approximately three hills of potatoes appeared in each yard of row. The plants were in bloom and about 45 cm. in height. At Wanatah, Indiana, the plot of 40 square meters was made up of nine square subplots with east to west rows. Three types of plots were available for measurements: tomato plants set at 90 cm. intervals, potato plants in conventional rows, and tomato plants set at 90 cm. intervals in conventional rows of potato plants. The plants were in bloom and about 45 cm. in height. Standard instrument shelters were located at the margin of the Clear Lake plot and in the center of the Wanatah plot, with the hygrothermographs located in them at a height of 40 cm. above the ground. The Ames measurements were taken on plants spaced at 90 cm. intervals.

Thermocouples were made of 30-gauge, enamel-coated, cotton-wrapped, copper and constantan wires. The thermocouples were formed by twisting the two bared wires together, heating them in an alcohol flame, and touching them to solder. The couples were then trimmed to a short, sharp, bright point of no greater diameter than the original twisted wires. The tempera-

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ture of the thermocouples was determined with a Brown electronic recording potentiometer capable of traveling 150° F (83° C) in five seconds and recorded on a chart scaled to 0.5° F. The temperatures of 16 different thermocouples were recorded in a period of 80 seconds. A sample record is shown in figure 1. The potentiometer recorded a soil-line temperature, number 5 in figure 1, of 107.2° F and stem temperatures, numbers 6 and 7, of 90.3 and 70.7° F in less than 15 seconds. In the presentation of results in this paper all readings were converted to the nearest 0.1° C.

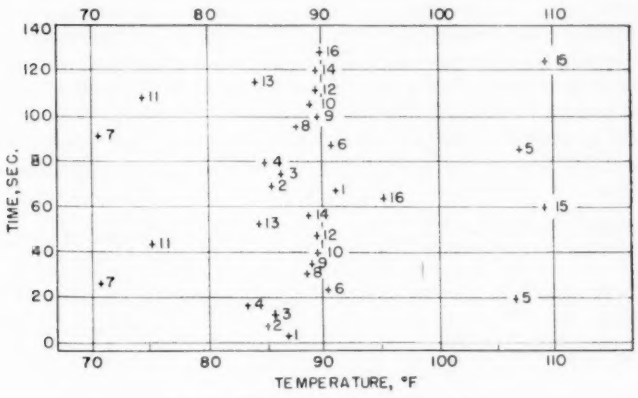

FIG. 1. Sample potentiometer record.

Observational methods and results

Widely different techniques have been employed in the measurement of leaf temperatures. Both SHREVE (**25, 26**) and EZEKIEL and TAUBENHAUS (**13**) measured leaf temperatures by methods which employed the use of rapid shading of the leaves. This procedure is inappropriate because a deciduous leaf has a thermal lag time of only about one half minute (**6, 33, 34**). MILLER and SAUNDERS (**21**) and EATON and BELDEN (**10**) attempted to observe leaf temperatures by clamping a thermocouple against leaves by means of a cork. CURTIS (**7**) found that leaf temperatures taken by this method were 0.9 to 3.7° C cooler than the temperature of a couple threaded into the leaf because the cork shaded the leaf. Many workers (**6, 7, 24, 32**) have measured leaf temperatures by threading a thermocouple into the leaf. EGGERT (**11**) has pointed out that it is best to have 7 cm. of the lead wire of the thermocouple under the same conditions as the junction itself. A thermocouple and a length of lead wire threaded into the leaf were expected to provide a close approximation of the leaf temperature, and this method was used here.

The manner of measuring air temperature also has varied. CURTIS (**7**) measured air temperature with a thermocouple held in the shade 2 cm. from a small wooden stake. He found that this method gave air temperatures

6 to 12° C cooler than if the thermocouple were shaded by a paper envelope as EATON and BELDEN (**10**) had done. CURTIS (**8**) found, also, that the observations of WALLACE and CLUM (**32**) taken by hanging a thermocouple in foliage gave physically impossible differences between air and leaf temperatures. The evaluation of the difference between leaf and air temperatures requires a precise measurement of both.

Because records were to be taken under both shaded and exposed conditions, it was necessary to evaluate the radiational error due to direct exposure of the thermocouple to insolation. The effect of full sunlight was estimated by a thermocouple alternately shaded and exposed to insolation at two locations. The first location was 1 cm. above an unshaded leaflet which was 38 cm. above bare soil on the outside row of the plot. The second location was in the free air 90 cm. laterally outside the plot and at a height of 38 cm. above bare soil. The results are given in table I. The temperature of the thermocouple in the free air was not increased by exposure to full sunlight, both shaded and unshaded thermocouples having mean temperatures of 26.2° C. The temperature of a thermocouple over a leaf was increased to 27.6° C by exposure of the thermocouple and leaf to full sunlight, in contrast to the mean temperature, 25.9° C, of a shaded thermocouple over a shaded leaf. Solar radiation and any long wave radiation absorbed by the thermocouple in the free air were evidently dissipated so rapidly by conduction that no significant warming of the thermocouple occurred. The warming concomitant with exposure to insolation of the thermocouple over a leaf could have been caused by three factors: reduced conduction, higher temperature of the surrounding air, and long wave radiation from the warm leaf. Because of the high reflectivity of most metals, little warming would be expected from the long wave radiation. The three factors would result in a decrease in the absolute magnitude of the temperature difference between a leaf and a thermocouple above it during either the day or night. Because of good conduction between a leaf and a thermocouple in contact with it and the small mass of the thermocouple in contrast to the large mass of the leaf, a thermocouple threaded into a leaf gives a

TABLE I

TEMPERATURES AT TWO DISTANCES FROM A POTATO LEAF AS DETERMINED BY A THERMOCOUPLE SHADED OR EXPOSED TO INSOLATION. HEIGHT 38 CM., FULL SUNLIGHT, JULY 6, 1950.

Exposure	Distance from leaf	Time replication			Mean
		1	2	3	
	cm.				*° C*
Exposed	1	26.8	27.9	28.2	27.6
Shaded*	1	26.1	26.0	25.7	25.9
Exposed	90	26.3	26.0	26.4	26.2
Shaded	90	25.9	26.4	26.4	26.2

*Leaf and thermocouple both shaded.

satisfactory measurement of leaf temperature while a thermocouple 1 cm. above the leaf gives an estimate of free air temperature that is biased upward during periods of strong incoming radiation and biased downward during periods of strong outgoing radiation. A thermocouple 1 cm. below a leaf would be expected to be biased in the same direction but to a smaller degree.

The temperature of a single leaflet was expected to vary with time. Because only one thermocouple could be inserted into each leaflet when several leaflets were examined, an auxiliary study was necessary to evaluate the temperature variation in a single leaflet. Curvature or irregularities of the lamina are a primary cause of temperature variation. ULLRICH and MÄDE (**29**) have shown that in an arched leaf a pool of cold air could be detected in the bowl formed by the leaf edges. CLUM (**6**) observed differences of 0.2 to 2° C between opposite sides of a leaf on a bright day. According to ULLRICH and MÄDE (**29**) very small differences exist between the temperature of top and bottom surfaces of sunlit leaves. Temperature measurements made at Ames on the top and bottom surfaces of sunlit tomato leaves gave values within 0.6° C of each other. Because of this small difference in the temperatures of the two surfaces, the leaf measurements presented here were restricted to the upper surface of leaves. To measure the temperature variation within a single leaflet, four thermocouples were threaded into one side of each of two upper, sunlit leaflets and two lower, shaded leaflets. The curvatures of the leaflets were small and they were nearly perpendicular to insolation. Twenty seconds were required for the determination of the temperature of the four thermocouples in each leaflet. From an analysis of variance of these data, the standard deviations of the temperatures within upper and lower leaflets were estimated to be the same, 0.4° C, although one would expect the variance of the temperature within an upper leaflet to be greater than in a lower leaflet because of the greater variation in radiation received.

THE ENERGY ECONOMY EQUATION

Leaf temperature relative to air temperature can be predicted from an energy economy of the leaf. The economy proposed is Energy from insolation + Energy from respiration = 2 × Energy consumed in transpiration + Energy consumed in photosynthesis + 2 × Energy exchange. The energy values for transpiration and exchange are doubled because two leaf surfaces per unit of leaf area participate in transpiration and exchange. Estimates of the order of magnitude of the components of the economy are made as follows.

Solar radiation is of the order of 1.0 cal./min./cm.^{2} direct radiation plus 0.2 cal./min./cm.^{2} diffuse radiation measured on a perpendicular surface (**2**). Receipt of energy varies from nearly 1.2 cal./min./cm.^{2} for a leaf perpendicular to the sun's rays to 0.3 cal./min./cm.^{2} for a slightly curved leaf set approximately parallel to the sun's rays. According to DINGER (**9**) and

SIDORIN (**27**) approximately one half of the incident insolation is absorbed by leaves. Thus the insolation component of the energy economy would be 0.60 and 0.15 cal./min./cm.^{2} for perpendicular and parallel leaves, respectively. Respiration does not contribute an appreciable quantity of energy (**15**), and its effect was disregarded. Transpirational water loss is of the order of 1 to 1.5 gm./hr./dm.^{2} (**1, 20, 21**). PENMAN (**22**) found the transpiration from a grass plot to be about 4 gm./hr./dm.^{2} In view of the greater amount of leaf surface per square centimeter of plot, his observations agree roughly with the estimate that transpiration from a sunlit leaf is 1 to 1.5 gm./hr./dm.^{2} This evaporation removes about 0.15 cal./min./cm.^{2} from the leaf and the economy component, $2 \times$ energy consumed in transpiration, is therefore also 0.15 cal./min./cm.^{2} for both surfaces of the leaf. Energy consumed in photosynthesis is of the order of 0.01 cal./min./cm.^{2} (**3, 28**). The small contribution of photosynthesis to the energy economy of the leaf was disregarded. Exchange of energy occurs by means of radiation and conduction. Energy exchange occurs between the leaf and the atmosphere, the soil, and nearby plants. This summation of radiation and conduction has been called emission (**3, 24, 33**) and Austausch (**15**). Because both emission and Austausch have clearly defined meanings in other fields, the general term exchange is used in this discussion. The number of calories dissipated per square centimeter per minute by exchange is the remainder after substitution of the five other components in the energy economy equation.

Leaf temperature relative to air temperature can now be predicted because exchange, E, is a function of the elevation of leaf temperature above air temperature. BROWN and ESCOMBE (**3**) related E empirically to the elevation of leaf temperature, T′, above air temperature, T. For the steady state they wrote: $E = H(T' - T)$ where the coefficient of exchange, H, is emissivity. BROWN and WILSON (**4**) estimated H to be: $H = 10^{-4}(119 + 174w)$ where w is wind speed in meters per minute. Since the estimate of the rate of cooling of leaves made by CLUM (**6**) and WATSON (**33, 34**) can be transformed[3] into estimates of H of the same order of magnitude as those of Brown and Escombe it was decided to use the following substitution in the energy economy:

$$2E = 2H(T' - T) = 2 \times 10^{-4}(119 + 1.74w)(T' - T)$$

The manner in which leaf temperatures are predicted is now apparent. The difference between energy gained from insolation and lost by transpiration by the leaf is attributed to exchange, and the exchange is related to elevation of leaf above air temperature by means of the above equation.

[3] The rate of cooling is a function of the lag time, L, of the leaf. L can be related to H as follows: $H = \frac{m}{S} \frac{c}{L}$, where m/S is mass per unit area and c is the specific heat of the leaf.

LEAVES PARALLEL OR PERPENDICULAR TO INSOLATION.—The energy economy of a leaf is given above as: Energy from insolation $-2\times$ Energy consumed in transpiration $=2\times$ Energy exchange. The appropriate values for a leaf perpendicular to insolation are $0.6-0.15=0.45$ cal./min./cm.^{2} for the sum of both surfaces of a leaf. For a leaf parallel to insolation $0.15-<0.15=+>0$ cal./min./cm.^{2} for the sum of both surfaces of a leaf. The energy consumed by transpiration from a leaf parallel to insolation will be less than 0.15 cal./min./cm.^{2} because the leaf will be cooler than the perpendicular leaf. The difference in exchange between leaves perpendicular and parallel to insolation corresponds to a difference of about 7.7° C between the leaves if the wind speed is 100 meters per minute. The value of H would be expected to vary between the two exposures due to differences in radiation and conduction exchange. Net loss by radiation exchanges to the atmosphere increases as a leaf is turned more toward the zenith (**14**). Net loss by conduction exchange is greater for a leaf facing the horizon (**23**). The result of these opposing factors is to decrease the expected temperature difference from 7.7° C to between 4.9 and 7.7° C.

TABLE II

TEMPERATURES OF UPPER AND LOWER POTATO LEAFLETS ORIENTED PARALLEL OR PERPENDICULAR TO INSOLATION, FULL SUNLIGHT, 1220 HOURS, JULY 6, 1950, CLEAR LAKE.

Height	Orientation	Space replication	Time replication				Mean
			1	2	3	4	
							°C
Upper	Perpendicular	a	29.8	28.8	28.8	28.9	28.8
		b	28.9	29.6	27.3	28.5	
	Parallel	a	25.6	25.4	24.9	25.7	25.6
		b	26.1	26.2	25.3	25.1	
Lower	Perpendicular	a	25.7	26.7	26.7	27.8	25.3
		b	23.0	23.9	24.4	24.3	
	Parallel	a	25.0	25.4	25.1	25.4	25.6
		b	25.5	25.8	25.8	26.2	

The observations of other workers tend to confirm this energy economy prediction. KONIS (**17**) found temperatures of leaves perpendicular to insolation were 3.3 to 7.3° C warmer than the temperatures of leaves exposed parallel to insolation. EATON and BELDEN (**10**) found a difference of 1.4 to 3.2° C.

A further test of the prediction was provided by the observations of temperatures of upper and lower potato leaves perpendicular and parallel to insolation, given in table II. The upper leaflets were exposed and the lower leaflets were shaded. The 3.2° C difference observed between upper leaves exposed perpendicular and parallel to insolation was not significantly different from the 4.9 to 7.7° C difference predicted from energy economies. Difficulty was encountered in maintaining the exposure of the leaves, par-

ticularly in the parallel exposure. Nevertheless, the error term was sufficiently small so that the 3.2° C difference was significantly different from zero. The angle of exposure of the lower shaded leaves did not affect the temperature. In the following investigations of leaf temperature, all measurements were taken with the leaves perpendicular to insolation.

Leaf temperature comparisons

The effect of exposure, time of day, cloud cover, crop and plant spacing upon leaf temperature was evaluated by comparing the temperatures of upper and lower leaves, temperatures of leaves at four times of day with clear and cloudy skies, temperatures of potato and tomato leaves, and temperatures of leaves in open and closed plantings.

Temperatures of upper and lower leaves.—Differences in temperature among upper and lower, shaded and exposed leaves were predicted from energy economies. If in the energy economy of sunlit upper and lower leaves we can assume equal insolation and 0.15 and 0.20 cal./min./cm.^{2} transpirational heat loss at the two levels, then we see from the energy economy proposed for a leaf perpendicular to insolation that the values of $2E = 2H(T' - T)$ are 0.45 and 0.40 cal./cm.^{2}/min. at the two heights. Because $(T' - T)$ is a function of H only, the difference between leaves at two levels on a plant can be predicted from a knowledge of the variation of H.

For a lower leaf, H is less than for an upper leaf because of lower net conduction and radiation exchange near the ground. Geiger (**14**) stated that for a wind of 100 meters per minute at the height of upper leaves a wind of 10 meters per minute occurred near the ground. The wind speeds of 100 and 10 meters per minute correspond to values of H of 0.029 and 0.013. These values of H correspond to elevations of upper and lower leaf temperatures over air temperatures of 7.5 and 15.4° C, respectively. The effect of different conduction exchanges at the two levels is considerable.

Loss of energy by radiation exchange from a lower leaf was expected to be less than from an upper leaf because the lower leaf is more nearly surrounded by objects at high temperatures than is the upper leaf. Consequently, a higher value of $(T' - T)$ is expected for the lower leaf than the 15.4° C predicted above from a consideration of conduction exchange only. The difference between upper and lower leaves would be expected to be $> 7.9°$ C.

The observed temperatures of upper and lower, sheltered and exposed leaves, table III, were employed to test the predictions. The lower leaves were almost on the soil line and the upper leaves were at a height of 45 cm. The lower exposed leaves were 4.4° C warmer than the upper exposed leaves. The difference of 4.4° C is significantly smaller than the predicted difference, 7.9° C, but is in the expected direction. As predicted, the upper and lower shaded leaves were at the same temperature. The lower exposed leaves were 12.3° C warmer than the lower shaded leaves. The difference of 12.3° C is significantly smaller than the predicted difference, $> 15.4°$ C,

but is in the expected direction. The predictions from the energy economy tended to be verified; lower exposed leaves were warmer than upper exposed leaves and both were warmer than shaded leaves.

EFFECT OF TIME OF DAY AND CLOUD COVER.—Energy exchange, E, was separated here into its components of radiation and conduction exchange and the energy economy of a leaf was expressed as: Energy from insolation (I) + Downward radiation exchange (D) = 2 × Energy consumed in transpiration (2W) + Upward radiation exchange (U) + 2 × Conduction exchange (2C). The first four components of this economy were estimated and the fifth component, 2C, obtained by difference. The term conduction as used here includes conduction and convection, with convection removing most of the heat from the vicinity of the plant. However, the heat carried away by convection must first get from the leaf to the air by conduction. The esti-

TABLE III

TEMPERATURES OF UPPER AND LOWER TOMATO LEAVES SHADED FROM AND EXPOSED TO INSOLATION, FULL SUNLIGHT, 0800 HOURS, JULY 27, 1950, WANATAH.

Exposure	Height	Space	Time				Mean
			1	2	3	4	
							°C
Exposed	Upper	a	32.0	32.6	31.8	31.5	31.2
		b	30.4	29.3	31.7	30.2	
	Lower	a	34.3	37.1	35.2	33.6	35.6
		b	35.2	36.7	36.1	36.6	
Shaded	Upper	a	22.8	23.7	22.8	23.5	23.4
		b	23.7	23.3	23.8	23.6	
	Lower	a	23.7	24.0	23.4	23.9	23.3
		b	22.9	22.6	23.3	22.8	

mation of the components is described in the following paragraphs and the results are tabulated in table IV.

Energy is gained by a leaf from insolation and downward radiation exchange. Insolation absorbed is estimated to vary from 0.60 on a clear day to 0.20 cal./cm.^{2}/min. on a day with 0.8 sky cover. Insolation is zero at night. The net gain by downward radiation exchange, D, depends upon the relative temperatures of the leaf and objects beneath it. On a clear day a leaf would gain < 0.10 cal./cm.^{2}/min.

Net losses of energy from a leaf occur by means of transpiration and upward radiation exchange. Transpiration, 2W, has been shown above to expend about 0.15 cal./min./cm.^{2} The loss decreases for a sheltered lower leaf or an upper exposed leaf on a cloudy day, and approaches zero at night. The net upward radiation loss from the leaf to a clear sky is given by an empirical formula suggested by BRUNT (**5**): $U = \sigma A^4(0.56 - 0.8e^{\frac{1}{2}})$, where σ is Stefan's constant, A absolute temperature, and e vapor pressure in millibars of the air at a height of 150 cm. LAUSCHER (**18**) has shown that

U is decreased to one half of the value for clear sky in the case of 0.8 cover of low clouds and to one fifth in the case of 1.0 cover of low clouds. For a temperature of 29.5° C and a vapor pressure of 16 millibars, U is 0.16 cal./min./cm.^{2} for zero cloud cover, 0.08 cal./min./cm.^{2} for 0.8 cloud cover, 0.03 cal./min./cm.^{2} for 1.0 cloud cover.

Energy lost by the leaf through conduction exchange, 2C, is estimated by difference of the estimates of the other components appearing in table IV. Loss of energy by conduction exchange can be related to excess of leaf temperature over air temperature as follows: $2C = 2K(T' - T)$, where K is the coefficient of conduction exchange. Therefore, positive values in the column of conduction exchange losses in table IV correspond to positive values of $(T' - T)$, *i.e.*, leaf warmer than air. The sign of the predicted values of

TABLE IV

CALCULATED ENERGY ECONOMY OF UPPER EXPOSED AND LOWER SHELTERED LEAVES. VALUES ARE CAL./MIN./CM.^{2} AND A POSITIVE SIGN INDICATES A NET LOSS OF ENERGY FROM THE LEAF.

Time	Sky cover	Exposure	Energy gain		Energy loss		Conduction loss	Predicted $(T' - T)$
			Insolation	Downward radiation	Transpiration	Upward radiation		
Day	0.0	Upper exposed	0.6	+< 0.1	+ 0.15	+< 0.2	+ 0.35	+
		Lower sheltered	0.0	+> 0.0	+< 0.15	0.0	− 0.15	−
	0.8	Upper exposed	0.2	+> 0.0	+< 0.15	+< 0.1	....	−
		Lower sheltered	0.0	+> 0.0	+< 0.15	0.0	−< 0.1	−
Night	0.0	Upper exposed	0.0	−> 0.0	> 0.0	+< 0.2	− 0.2	−
		Lower sheltered	0.0	+> 0.0	0.0	0.0	+> 0.0	+
	1.0	Upper exposed	0.0	−> 0.0	> 0.0	+> 0.0	−> 0.0	−
		Lower sheltered	0.0	+> 0.0	0.0	0.0	+> 0.0	+

$(T' - T)$ is shown in table IV. For an upper sunlit leaf the positive conduction exchange corresponds to an elevation of leaf temperature above air temperature. Similar reasoning applied to the negative conduction exchange losses, *i.e.*, energy gains, in table IV lead to the prediction that leaf temperatures are less than air temperatures for lower sheltered leaves during the day, clear or cloudy, and the upper exposed leaves during the night, clear or cloudy, and during a cloudy day. The small positive conduction exchange losses for lower sheltered leaves at night lead to the prediction that leaf temperatures are warmer than air temperatures for lower sheltered leaves at night, clear or cloudy.

The above predictions were tested by comparing them with temperature observations taken by thermocouples threaded into or supported 1 cm. above upper exposed and lower sheltered tomato leaves at Wanatah on July 25,

26, and 27, 1950. Observations were made under clear and cloudy skies at 0400, 1000, 1600, and 2200 hours. The clouds during the day, 1000 and 1600 hours, were swelling cumulus that provided about 0.8 coverage. The clouds at night formed an overcast. The differences observed between the temperatures within the leaf and 1 cm. above it are shown in table V. Exchange of energy between the leaf and the thermocouple above it and departure of the thermocouple from the air temperature due to radiation tend to decrease the absolute magnitude of the temperature difference. The differences observed may be erroneously small, but the sign of the difference should be unaffected and the observations of table V are used to test the predictions of table IV. Supplemental data taken at Ames in August and September of 1951 with thermocouples threaded into leaves or in a nearby instrument

TABLE V

TEMPERATURES OF THERMOCOUPLES THREADED INTO OR SUPPORTED 1 CM. ABOVE TOMATO LEAVES.

Sky cover	Leaf height	Location of thermocouple	Day temperature	Night temperature
			°C	°C
Clear	Upper	In leaf	32.8	10.4
		Above leaf	30.5	11.0
		Difference	+2.3	−0.6
	Lower	In leaf	26.6	11.7
		Above leaf	27.2	11.1
		Difference	−0.6	+0.6
Cloudy	Upper	In leaf	24.1	17.0
		Above leaf	24.7	17.4
		Difference	−0.6	−0.4
	Lower	In leaf	23.8	17.4
		Above leaf	24.1	17.0
		Difference	−0.3	+0.4

shelter provide additional tests of the prediction. These data are summarized in table VI.

The observed excess of leaf temperature over air temperature given in table V for a sunlit upper leaf, 2.3° C, agrees with the positive value of $(T' - T)$ predicted in table IV. It agrees also with the 3.2° C difference given in table VI. Differences as large as 8° C were measured under very clear, calm conditions. This observation agrees also with the data of others (**6, 7, 12, 29**). The observations, table V, that lower sheltered leaves on clear and cloudy days and upper exposed leaves on clear and cloudy nights are cooler than the air agrees with the negative value of $(T' - T)$ predicted in table IV. This observation agrees also with those of others (**12, 29**). The observed small excess of leaf over air temperature for lower sheltered leaves on clear and cloudy nights agrees with the positive value of $(T' - T)$ predicted. Upper exposed leaves on a cloudy day were observed to be cooler than the air. This observation agrees with those of CURTIS (**7**).

Energy economy predictions were thus verified by direct observations and determinations reported in the literature.

The temperature relations between upper exposed and lower shaded leaves can be obtained from the observations of tables V and VI. The excess of the temperature of upper exposed over temperature of lower sheltered leaves is 6.2° C under clear sky and 0.3° C under cloudy sky during the day and −1.3° C under clear sky and −0.4° under cloudy sky during the night. The temperature of upper exposed leaves, which was about 4.6° C higher than the temperature of lower sheltered leaves on clear days, approached the temperature of the lower sheltered leaves on a cloudy day. The temperature of upper exposed leaves, which was about 1.3° C lower than that of the lower sheltered leaves on clear nights, approached the temperature of the lower sheltered leaves on a cloudy night.

TABLE VI

TEMPERATURES OF THERMOCOUPLES THREADED INTO A LEAF OR LOCATED IN A NEARBY INSTRUMENT SHELTER.

Sky cover	Leaf height	Location of thermocouple	Day temperature	Night temperature
			°C	°C
Clear	Upper	In leaf	25.9	14.0
		In shelter	22.7	14.8
		Difference	+3.2	−0.8
	Lower	In leaf	21.9	....
		In shelter	22.7	....
		Difference	−0.8	....
Cloudy	Upper	In leaf	21.1	16.6
		In shelter	21.9	17.4
		Difference	−0.8	−0.8
	Lower	In leaf	21.4	....
		In shelter	21.9	....
		Difference	−0.5	....

TEMPERATURE OF POTATO AND TOMATO LEAVES.—No difference in any of the components of the energy economy could be found between the leaves of potato and tomato when the plants were grown under similar conditions. Therefore, equal leaf temperatures were predicted for the two species, although EATON and BELDEN (10) reported a temperature difference between the leaves of two varieties of cotton. To test the prediction, measurements were taken of potato and tomato leaf temperatures in an interplanted plot at Wanatah. The foliage of the two species was intertwined at the time of observation. The trivial differences, about 0.3° C, observed between similarly exposed leaves of the two species were not significant.

TEMPERATURES OF TOMATO LEAVES IN OPEN AND CLOSED PLANTINGS.—The effects of differences in radiation exchange and ventilation between open and closed plantings were considered in order to predict any differences in leaf temperatures. A larger bare soil surface provides greater radiation from the soil to the leaves in an open planting. However, increased venti-

lation in open plantings gives a higher coefficient of conduction and higher transpiration (**18**) than in the closed planting. Because increased energy loss through conduction and transpiration tends to counteract the increased energy gain through radiation exchange in the open planting, no temperature difference was predicted between the two types of plantings. Observations made at Wanatah showed only trivial differences between similarly exposed leaves in open and closed plantings. If plants of equal size are set at intervals rather than in continuous rows, *i.e.*, open rather than closed planting, a greater proportion of the leaves are exposed to insolation. Therefore, the mean leaf temperature of open-spaced plants would be higher during the day than that of close-spaced plants because exposed leaves are warmer than shaded leaves during the day despite the fact that similarly exposed leaves are at the same temperature in the two types of plantings.

Relation between leaf and instrument shelter temperatures

Different exposures of thermocouples or thermometers have been used in estimating air temperatures in a plant cover (**8, 10**). To obtain a measure of the difference between leaf and air temperature, leaf temperatures were compared with temperatures in a nearby instrument shelter. An upper exposed leaf on a clear day at Wanatah was found to be 4.4 to 6.7° C warmer than the thermograph temperature registered in an instrument shelter. At Ames in 1951, an upper exposed leaf on a clear day was found to be 3.2 to 8.2° C warmer than the thermocouple temperature registered in an instrument shelter. The upper leaf was 1 to 1.5° cooler on a cloudy day, 1 to 3° cooler on a clear night, about 1° C cooler on a cloudy night than temperatures measured either with a thermograph or thermocouple in an instrument shelter.

On a clear, windy day thermocouple temperatures 1 cm. above a leaf exposed to radiation were 1.5 to 2.5° C warmer than a thermocouple in an instrument shelter, and a thermocouple 1 cm. below an upper exposed leaf was about 1° C warmer than the thermocouple in the shelter. Under such conditions thermocouples close to leaves exposed to radiation gave values above air temperature. Under calm conditions this difference would be expected to be greater, because of the greater warming of the air near the leaf. On cloudy days, and on clear and cloudy nights, temperatures 1 cm. below an upper leaf were within 0.5° C of temperatures in the instrument shelter.

Summary

Earlier proposals for the energy economy of leaves were integrated into forms which could be used to predict the difference in temperature between leaf and environment. The proposed economy is tested by comparing the predictions with observations in the field.

The method of temperature observation was evaluated. The effects of several factors upon leaf temperature were predicted and verified. The factors considered were changes in radiation due to time of day, sky cover,

angle of incidence and shading; changes in ventilation due to location on the plant and density of stand; and differences in plant species.

A leaf perpendicular to insolation was 3.2° C warmer than a leaf parallel to insolation. Shaded leaves on all parts of the plant had nearly equal temperatures while leaves exposed to insolation at the top of the plant and near the soil line were 7.8 and 12° C, respectively, warmer than the shaded leaves.

Upper exposed leaves were 3.3 to 8° C warmer on clear days and 0.8° C cooler on cloudy days than the air temperature in an instrument shelter. At night the same leaves were 0.5° C cooler than the air in the shelter. Lower sheltered leaves were 0.5 to 0.8° C cooler during the day than temperatures in an instrument shelter. On a cloudy night lower sheltered leaves were 0.5° C warmer than the air 1 cm. above them.

Air temperatures 1 cm. below an exposed upper leaf were about 1° C warmer than temperatures in an instrument shelter on a clear, windy day. No temperature differences were observed between similarly exposed potato and tomato leaves or between similarly exposed tomato leaves in open and closed plantings.

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MALONIC ACID AS AN INHIBITOR OF MAIZE ROOT RESPIRATION

H. Beevers

(WITH SIX FIGURES)

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Early experiments on the effects of malonate on plant respiration led to contradictory results which were largely resolved by the work of Bonner and Wildman (**3**), Turner and Hanly (**18**), and Laties (**8**) who showed that pH conditions affected the response to the inhibitor. It is now clear that for some plant tissues at least, under appropriate conditions of pH and malonate concentration, O_2 uptake is retarded and accumulation of succinic acid occurs (**2, 7**). Other evidence (**5**) has also been obtained which supports the view that the oxidation of pyruvate in plants occurs through a cycle which can be slowed down by malonate, but there are no records of the accumulation of other compounds in the presence of the inhibitor.

The present paper describes experiments in which changes in respiratory quotient (R.Q.) and the associated accumulation of some other cell components (not regarded as precursors of succinate) were followed at various malonate concentrations, as well as experiments in which the relationship between pH and the inhibitions induced by malonate was re-examined.

Methods

Hybrid corn, Wf9 × 38–11, was soaked in tap water overnight with Semesan as a fungicide, washed, drained, and placed in the dark in layers of 1 cm. thickness in large beakers. After three days at 23 to 26° C in contact with a minimum amount of water, the roots of the germinating corn had bound the grains together in mats which were then supported on small beakers placed inside the original ones and well watered. During the next two days, the roots grew rapidly downwards away from the grain; and from these roots, tips about 15 mm. long were detached, washed in distilled water, and counted into samples. In the experiments reported here, 20 root tips with phosphate buffer (0.06 M) were used in each flask; such samples had a dry weight of about 16 mg. and the average O_2 uptake per hour at 25° C was 70 cu. mm. (Q_{O_2} value of 4.4). The duration of most of the experiments was three to five hours, although other experiments showed that the O_2 uptake continued at a steady rate for considerably longer periods than this (fig. 4). It should be mentioned that the material respired equally rapidly in distilled water as in the buffered medium; there was no marked salt-induced respiration (**18**). Changes in pH in the range of 4.0 to 8.0 have very little effect on the respiratory activity; even at pH 2.9, the respiration rate was maintained at slightly over 80% of the rate at pH 6.0.

Malonate solutions were adjusted to the pH at which the individual experiment was to be carried out before making the solutions up to volume.

For experiments in which alcohol and acetaldehyde were to be measured, samples of 150 root tips with buffer and a known amount of malonate were placed in large boiling tubes and aerated. The outgoing gas-stream was passed through 2% bisulphite (**11**), and the amounts of acetaldehyde trapped were estimated from time to time in 1-ml. samples by the method of STOTZ (**14**). The alcohol estimation procedure of WILLIAMS and REESE (**20**) was applied to aliquots of a steam distillate obtained from the roots and the surrounding solution made alkaline with KOH. It was first ascertained that more than 95% of the alcohol was recovered from a solution of known concentration in the first 15 ml. of distillate; this amount was collected in each case and 5-ml. aliquots were used for the estimation, using

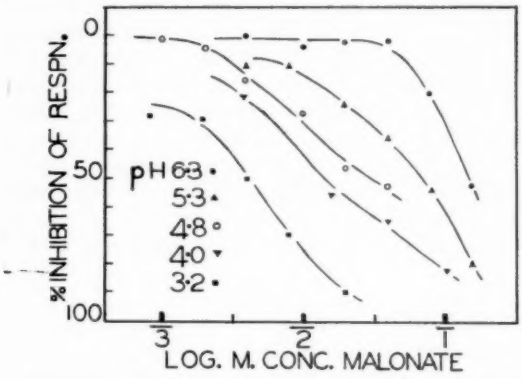

FIG. 1. Respiratory inhibitions induced by graded dilutions of malonate at five different pH levels. The points are O_2 uptake in the period of two to three hours expressed as per cent. of controls at the same pH.

half the quantities of reagents and a dichromate mixture as described by Williams and Reese. The addition of malonate or succinate in high concentrations before distillation did not alter the amount of alcohol distilling from a known sample.

Results

THE EFFECT OF pH ON MALONATE INHIBITION

In figure 1 are plotted the results of a series of experiments at pH 3.2 to 6.3 in which graded dilutions of malonate at the pH of the buffer were added to samples of roots. The per cent. inhibition was evaluated by comparing the respiration of such samples over the period of 120 to 180 minutes with that of controls at the same pH. The fact that the rates of O_2 uptake were virtually constant after the roots had been in contact with the malonate for about 90 minutes (fig. 4) justifies the adoption of this time interval.

It will be seen that a series of more or less parallel curves is obtained, and that, as the pH is raised, the curves are displaced to the right. No inhibition was obtainable at pH 6.3 until concentrations of 0.03 M were exceeded, whereas at pH 3.2 the curve was already in the descending region at 0.001 M. If the concentrations which induce a given inhibition, say 50%, are compared, there is an increase of some 50 times as the pH is raised from 3.2 to 6.5 (fig. 2). The results also show that for any given concentration of inhibitor, it is the pH which determines the extent of the inhibition, reductions in pH bringing about increases in the amount of inhibition. Thus when the per cent. inhibition induced by a given concentration of malonate is plotted against pH, a sigmoid curve is obtained, falling from zero inhibi-

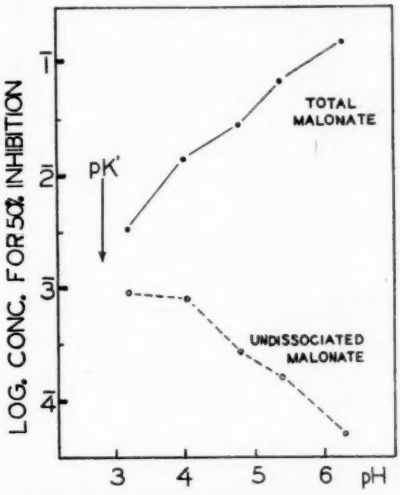

FIG. 2. The relationship between pH and the amount of malonate required to induce 50% inhibition of O_2 uptake. The continuous line shows the total applied malonate, and the points on the broken line show the calculated concentrations of undissociated acid corresponding to the applied malonate.

tion at a given pH to almost complete inhibition at a pH value a few units lower. It should be clear from figure 1 that if a series of malonate concentrations were chosen and the results plotted in this way, a series of more or less parallel curves displaced along the pH axis would result; the inhibition observed with the highest malonate concentration would be at the greater pH values. This point is made here because the placement of such a single sigmoid curve along the pH axis and the chance coincidence of its point of inflection with that of the dissociation curve for the second H of malonic acid (pH 5.3) has been interpreted by BONNER and WILDMAN (3) as indicating that the monobasic malonate ion is the major cell penetrant. Direct evidence against the monovalent ion hypothesis is given by the fact that for

any concentration of malonate, reductions in the pH below 4.5 (which would lead to a reduction of the concentration of monobasic malonate ions) induce not smaller but greater inhibitions than those at pH 4.5. The results recorded here for malonate are in good accord with those described in earlier studies (**1, 12, 13**), and there is no reason to suppose that the penetration behavior of this weak acid is markedly different from others investigated. The close similarity of the data shown in figure 2 (in which the concentrations of malonate inducing 50% inhibition are plotted against pH) to results previously described supports this view. The general interpretation of such

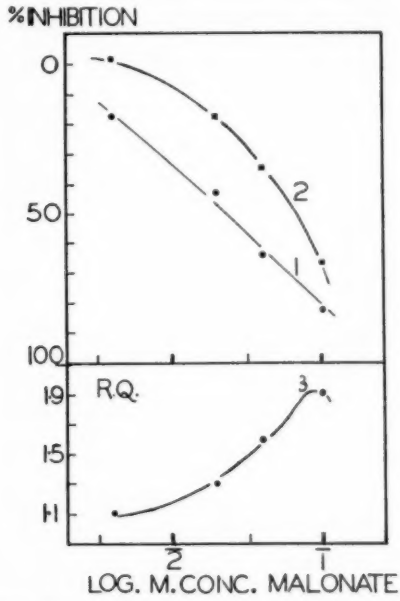

FIG. 3. The effect of malonate in different concentrations on O_2 uptake (curve 1), CO_2 output (curve 2), and R.Q. during the period of two to three hours (curve 3).

curves is that the response to a weak acid is determined largely, but not entirely, by the concentration of undissociated molecules present. The fact that the broken line in figure 2 (joining points which show the concentrations of undissociated molecules corresponding to the equi-effective concentrations of applied malonate) does not remain horizontal at pH levels higher than pK (**1, 12, 13**) bears out this statement. Subsequent experiments were carried out at pH 4.0.

EFFECT OF MALONATE ON RESPIRATORY QUOTIENT

Most workers on the effects of malonate have confined their attention to O_2 uptake although reports have been made of increases in R.Q. due to malo-

nate treatment (**3, 8**). Figure 3 shows the effects of a series of malonate concentrations on the O_2 uptake and CO_2 output of samples of roots during the period of 120 to 180 minutes after addition. At each concentration, the effect on CO_2 uptake is less than on O_2 uptake; and the R.Q. is progressively raised from the control value of 0.9. It will be seen that, although the R.Q. is highest at about 0.1 M malonate where it may be initially as high as 2.6, the malonate concentration which induces the greatest excess of CO_2 in absolute terms is somewhat lower than this, *i.e.*, where the O_2 uptake is inhibited by some 50%.

It will be apparent that if malonate inhibits aerobic respiration because of a specific interference with succinic dehydrogenase, there is no reason to suppose that anaerobic respiration would be affected since the enzyme is not

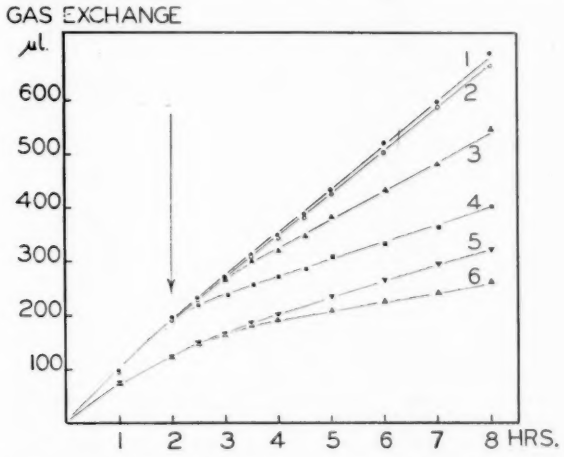

FIG. 4. The progress of aerobic O_2 uptake (curves 1 and 4) and CO_2 output (curves 2 and 3) and anaerobic CO_2 output (curves 5 and 6). Malonate (0.04 M) was added to root samples for curves 3, 4, and 6 after two hours (indicated by arrow).

involved in the latter process. However, malonate in high concentrations (but no higher than have frequently been used to show aerobic inhibition) can bring about extensive inhibition of respiration in nitrogen. The results of an experiment in which 0.04 M malonate was added after two hours to root samples respiring in air and in nitrogen are shown in figure 4. It will be seen that, in air, the control rates of CO_2 output and O_2 uptake continued unchanged with an R.Q. of 0.97, while the rate of O_2 uptake with malonate was reduced by 60% and that of the CO_2 output with malonate was reduced by some 27% (R.Q. 1.8). However, the rate of anaerobic CO_2 production was also reduced considerably by this same concentration of malonate. The rate of CO_2 output in N_2 by the control was about half of the rate of CO_2 production in air, but this rate was reduced by 40% in

the presence of malonate. Figure 5 shows the results of an experiment in which different concentrations of malonate were added to systems in nitrogen and the percentage inhibitions evaluated over controls during the period of two to three hours after addition (curve 1). Curve 3 shows the effects of the same malonate concentrations on aerobic respiration (O_2 uptake) over the same time period. It is clear that those concentrations of malonate which bring about large inhibitions of aerobic respiration are also effective in reducing the rate of respiration in N_2. Curve 2 shows that there was no marked difference in the extent of the effect on aerobic respiration whether or not aerobic respiration was proceeding during the development of the inhibition.

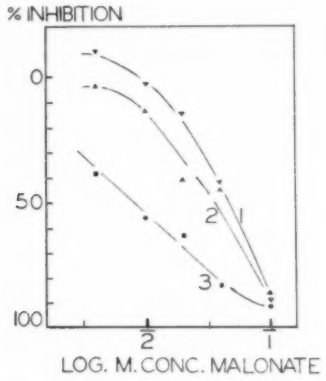

FIG. 5. The effect of different concentrations of malonate on CO_2 output in N_2 with malonate added in N_2 (curve 1), CO_2 output in N_2 with malonate added previously in air (curve 2), and O_2 uptake in air (curve 3).

THE INDUCTION OF AEROBIC PRODUCTION OF ACETALDEHYDE AND ALCOHOL

The fact that R.Q. values were maintained at values considerably greater than unity when roots were treated with concentrations of malonate which reduced O_2 uptake suggested the possibility that the inhibitor was inducing aerobic fermentation. When it was further observed that strong iodoform reactions were obtained after large samples of roots (several hundreds) had been aerated in 0.04 M malonate, the quantitative nature of the production of acetaldehyde and alcohol was investigated. Figure 6 shows the results of experiments in which acetaldehyde trapped from the outgoing air stream and alcohol from a distillate of roots and surrounding liquid were estimated after samples of 150 roots had been aerated in contact with various concentrations of the inhibitor for three and one half hours at pH 4.0. Only trace amounts of acetaldehyde and alcohol were produced in control systems without malonate, and the values refer to the excess of acetaldehyde and

alcohol over such controls, which were used as blanks in the final color analysis. It is clear that as the concentration of malonate is increased beyond 0.003 M there is a striking and regular increase in the amount of acetaldehyde recovered. The corresponding curve for alcohol shows again a sharp rise beginning at a concentration of 0.003 M, but at 0.02 M a peak is reached and thereafter the curve declines. Thus concentrations of malonate which inhibit O_2 uptake and induce high R.Q. values also bring about the aerobic production of acetaldehyde and alcohol in substantial amounts. It will be seen that the total carbon loss due to acetaldehyde and alcohol also reaches a peak at 0.02 M and declines at the highest malonate levels; this fact is in accord with the observation that the greatest excess of CO_2 in manometric experiments was found at this concentration of malonate (fig. 3). When 0.1 M malonate was applied to roots in nitrogen, the alcohol and acetaldehyde accumulations over a period of four hours were reduced by

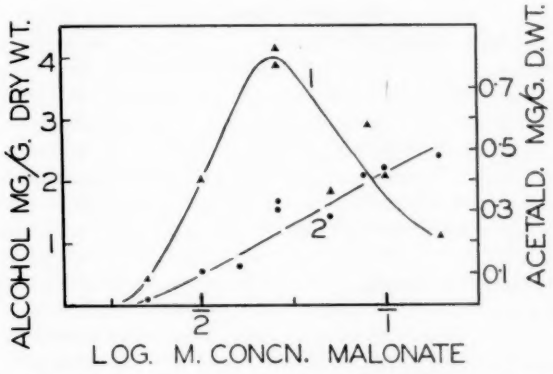

FIG. 6. The accumulation of alcohol (curve 1) and acetaldehyde (curve 2) in a three and one half hour period at different concentrations of malonate.

33 and 37% respectively and the alcohol/acetaldehyde ratio (38) remained near the control level of 43. Malonate, therefore, has no direct effect on the equilibrium between alcohol and acetaldehyde. In air, however, the amounts of acetaldehyde produced are such that the ratio of alcohol/acetaldehyde is lower than 40 : 1 and, as figure 6 shows, this ratio is drastically reduced as the malonate concentration is increased. Indeed, the rate of acetaldehyde production after the first few hours in contact with the inhibitor may far exceed that of the production of alcohol. Table I shows the results of an experiment in which samples were taken for alcohol analysis after three hours and again after eight hours in contact with the inhibitor. It will be seen that in the case of the lower malonate concentration both alcohol and acetaldehyde increased with time; the alcohol continues to exceed the acetaldehyde but by a ratio much lower than 40 : 1. When 0.09 M malonate was present, however, there was a large amount of alcohol

TABLE I

THE AEROBIC ACCUMULATION OF ALCOHOL AND ACETALDEHYDE IN THE PRESENCE OF MALONATE (DRY WEIGHT BASIS).

	Accumulation after three hours		Accumulation between three and eight hours	
	Alcohol	Acetaldehyde	Alcohol	Acetaldehyde
	mg./gm.	*mg./gm.*	*mg./gm.*	*mg./gm.*
0.03 M malonate	1.23	0.11	1.77	0.16
0.09 M malonate	3.2	0.41	0.08	0.63

produced in the first three hours, but only a relatively small amount during the second period, during which the evolution of acetaldehyde was maintained; the ratio during this period was very low (0.13).

Discussion

Malonate, as a competitive inhibitor of succinic dehydrogenase is frequently regarded as one of the most specific inhibitors; and in work with animal tissues, in which the normal content of succinic and other acids is low and strong inhibition (greater than 70%) can be achieved in concentrations as low as 0.001 M, this view is justified (**6**). However in work with plant tissues, in which greater amounts of these acids occur, it is noteworthy that considerably larger amounts of malonate must be applied even at suitable pH levels to induce similarly large responses; and some authors have questioned the specificity of the inhibitor at these high levels (**4, 19**). The results presented here also place doubt on its absolute specificity, in so far as some inhibition of the anaerobic respiration of maize tissue results when malonate is applied in concentrations which have often been used to demonstrate the inhibition of aerobic respiration. The maximum inhibition of aerobic respiration obtainable without, at the same time, inducing some inhibition of anaerobic respiration was 30 to 40%. As the malonate concentration was increased, aerobic respiration continued to decline but anaerobic respiration also was increasingly affected; and it therefore appears that only those inhibitions of aerobic respiration induced by concentrations which do not reduce anaerobic respiration can be wholly ascribable to a specific effect of malonate on the succinic dehydrogenase. In maize roots in air, as in most other plant tissues, it may be presumed that the rate of removal of pyruvate along the aerobic path is sufficiently rapid to prevent its diversion to alcohol *via* acetaldehyde, since these materials cannot ordinarily be detected in any but trace amounts. The fact that alcohol and acetaldehyde do appear in substantial amounts in the presence of malonate might be interpreted as indicating that the breakdown of pyruvate by the cycle is sufficiently reduced to induce its accumulation and consequent diversion towards alcohol. However, when O_2 uptake is strongly inhibited, the CO_2 output, although not so greatly reduced, still falls short of the rate expected if glycolysis was unaffected and all of the unused pyruvate were

being so diverted, and it is in fact clear (fig. 5) that under these conditions one or more of the anaerobic events are also inhibited. As the concentration of malonate is increased from zero, the accumulation of acetaldehyde and alcohol increases but at higher concentrations, as anaerobic events become progressively slower, the curve declines (fig. 6).

In this connection, it is noteworthy that in experiments on the accumulation of succinate in the presence of the inhibitor (**7**), the amounts of accumulant were not related to the amount of added malonate in a way which might have been expected if the sole effect was on the succinic dehydrogenase; at the highest malonate levels, there was only a small increase over the control level of succinate. It is clear then that there are additional points in the respiration sequence which are malonate sensitive; and although these can only be surmised at present, it seems likely that at least one point is a glycolytic step common both to anaerobic and aerobic respiration.

While malonate inhibits anaerobic respiration, it does not change the relative amounts of acetaldehyde and alcohol accumulated. The much lower ratios of alcohol/acetaldehyde, which may approach zero, when the tissue is inhibited in air are therefore not due to a direct influence on the alcohol dehydrogenase. It is clear that the reduction of acetaldehyde to alcohol would require a continuous supply of diphosphopyridine nucleotide, and it seems likely that the reaction is slowed down because the acetaldehyde-alcohol system cannot compete successfully for the reduced coenzyme with the aerobic oxidation mechanism. A similar explanation would account for the low alcohol/acetaldehyde ratios which have been observed in unpublished experiments by the author when the level of pyruvate was artificially increased in air by adding it to the medium or when dinitrophenol was added in suitable amounts.

For root tissues of maize, malonate may be added to the list of respiratory inhibitors including terminal oxidase inhibitors such as HCN, azide, CO, and H_2S (**10, 17**) and also high concentrations of CO_2 (**15**) which induce aerobic fermentation or zymasis (**15, 16**). It is not known to what extent such production of acetaldehyde and alcohol has been a feature when malonate has been applied to other plant tissues in air, but some recorded high R.Q. values suggest that the phenomenon may be of general occurrence. When the possibility of inhibition of reactions other than that controlled by the succinic dehydrogenase is also considered, it will be apparent that there are good reasons for supposing that strong inhibitions by malonate will not readily be reversed by supplying succinate. It has not been found possible to induce such reversal in maize tissue (*i.e.*, a recovery when succinate was added after the development of the inhibition) which is in accord with the experiences of some authors (**9, 18**), although some protective action of succinate against malonate added subsequently has been demonstrated.

Summary

The relationship between the pH of the medium and the efficiency of malonate as a respiratory inhibitor has been re-examined. The results show that in this respect the behavior of malonate is similar to that of other inhibitors which are weak acids; there is no evidence that it penetrates predominantly as the monobasic ion.

As the concentration of malonate was raised, both CO_2 output and O_2 uptake were increasingly inhibited, but the former was relatively less strongly affected so that the R.Q. rose steadily from the control value. As the malonate concentration was raised to levels beyond 0.01 M, anaerobic as well as aerobic respiration was increasingly inhibited.

Malonate in high concentrations in nitrogen did not alter the alcohol/acetaldehyde ratio, although the rate of anaerobic respiration of the roots was diminished. Roots whose aerobic respiration was inhibited by malonate produced acetaldehyde and alcohol in amounts and in a proportion determined by the concentration of the inhibitor. At high levels of malonate, the rate of alcohol production was greatly reduced while acetaldehyde production continued.

The results are interpreted as indicating that in maize root tissue there are at least two distinct effects of malonate; those concentrations which strongly reduce O_2 uptake in air also slow down certain of the anaerobic events. The origin of the acetaldehyde and alcohol is considered to be some of the pyruvate which is not used when the cyclic break-down system is inhibited by malonate. Some implications are discussed.

The author wishes to express his thanks to Mr. M. F. Oberbacher of this department for his cooperation during the early part of the work reported here.

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ASCORBIC ACID FORMATION IN POTATO TUBER SLICES[1]

FREDERICK G. SMITH

(WITH ONE FIGURE)

Received January 7, 1952

Introduction

The occurrence of ascorbic acid in plants and the environmental, nutritional and genetic factors involved have been widely studied but little is known about the actual mechanism of its formation. Previous work on this problem has been chiefly with germinating seedlings (**15, 16**) or leaf tissue (**1, 24, 25**) in which photosynthesis or rapid growth also occurred. The accumulation of ascorbic acid in potato tuber tissue, pointed out by GUTHRIE (**11**), STEWARD and PRESTON (**26**), PROKOSHEV (**19**), and others, indicated, therefore, that this might be favorable material for studying ascorbic acid synthesis, especially in relation to respiration.

Sugars have most frequently been suggested as precursors of ascorbic acid in plants (**1, 6, 15, 20**) and there is evidence relating the process to both photosynthesis (**24, 25**) and respiration (**11, 19, 26**). However, the most concrete suggestion for a mechanism of ascorbic acid synthesis has come from the work of SMYTHE and KING (**23**) who showed that certain glycolytic intermediates may increase synthesis in rat tissues.

This paper reports (a) a technique for comparing ascorbic acid synthesis and respiratory capacity in potato tuber slices, (b) further identification of the ascorbic acid formed, and (c) evidence that glycolysis is involved in the formation.

Materials and methods

A number of lots of potatoes of several varieties and sources were tested for ability to synthesize ascorbic acid in the slice system described below. Some were not sufficiently active when first tested or soon lost their ability in storage. Several lots, however, showed good synthetic ability which was maintained in storage at 10 to 15° C for four to six months.

Tuber slices, 8 mm. in diameter and 0.4 mm. in thickness, were sliced on a sliding microtome from a cube in which four or five cylinders were cut by a sharp cork borer and left *in situ* to brace the tissue. The slices were mixed and washed repeatedly till starch grains from the cut cells were no longer visible in the wash water. In this way large batches of uniform slices were readily prepared which were well adapted to chemical and manometric measurements with minimum sampling error.

Ascorbic acid synthesis was measured in systems of 100 to 200 slices incubated in 100 ml. of distilled water in 250 ml. centrifuge bottles in a 30° C bath. Inhibitor solutions which were added were adjusted to pH 6 with

[1] Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper no. 879, December 14, 1951.

KOH. Filtered air was bubbled through each bottle in series at a controlled rate to give thorough but gentle aeration and stirring. The suspending medium was changed daily or oftener if turbidity appeared. Under these conditions methylene blue smears showed no visible bacterial growth during the incubation period. Before chemical or manometric analysis, slices were rinsed and blotted lightly on filter paper.

Duplicate samples of 20 slices each were analyzed for ascorbic acid by each of two methods, the indophenol-xylene method of ROBINSON and STOTZ (**21**) and the dinitrophenylhydrazine method of ROE and KUETHER (**22**). In the first method, the tissue was homogenized in 2 ml. of 4% metaphosphoric acid–1 *N* sulphuric acid using a glass tissue grinder and the suspension was washed into a test tube with 2 ml. of acetate buffer. One to 4 ml. of 0.011% indophenol dye was then added and the excess dye extracted as quickly as possible with 10 ml. of redistilled xylene. Another slice sample was used to determine non-formaldehyde-bound reducing material by adding 1 ml. of 40% formaldehyde after the buffer and incubating eight minutes at room temperature before the dye reaction. Tests also were made for bound ascorbic acid by refluxing the extracts for various periods before adding the buffer.

In the dinitrophenylhydrazine method for total ascorbic acid (reduced plus dehydro-) the slices were homogenized in 5 ml. of 5% metaphosphoric acid and the ascorbic acid oxidized with 0.01 *N* iodine-iodide to a permanent starch-iodine blue color. Then 50 mg. of thiourea was added quickly and the solution stirred and centrifuged. Duplicate 2-ml. aliquots of the supernatant were transferred to test tubes, one of which contained 0.5 ml. of the dinitrophenylhydrazine reagent. Both tubes were stoppered and incubated at 37° C overnight. After cooling the tubes in an ice bath, 2 ml. of 85% sulphuric acid was added slowly to each tube and 0.5 ml. of the reagent to the blank tube. Both tubes were then stirred thoroughly and held at room temperature for 30 minutes before reading the transmission at 540 millimicrons in a Beckman spectrophotometer. Separate determinations of dehydroascorbic acid were made on slices homogenized in 5% metaphosphoric acid–1% thiourea and analyzed as above without oxidation.

With both methods, recovery of ascorbic acid added to homogenates was complete within the precision of the technique. The coefficient of variation for duplicate analyses averaged about 5% for both methods and for determinations before and after incubation.

Measurements of respiratory capacity were made in a standard Warburg apparatus using duplicate flasks containing 20 slices in a 3 ml. volume of 0.02 M pH 5.7 phosphate buffer for each determination. Oxygen uptake (Q_{O_2}) was measured in an oxygen atmosphere in which rates were shown not to be limited by gas diffusion in slices from 0.2 to 0.5 mm. in thickness. Anaerobic carbon dioxide evolution ($Q^{N}_{CO_2}$) was measured in nitrogen and aerobic carbon dioxide evolution ($Q^{O}_{CO_2}$) in oxygen by the direct method (**7**) with correction for change in bound carbon dioxide in both cases. Under these conditions all three rates were linear for at least the one hour du-

ration used. Where inhibitors were present during slice incubation the same concentrations were used in the Warburg flasks. All rates are expressed as microliters per hour per 20 slices.

Results

Ascorbic acid synthesis was dependent on adequate oxygen supply. If the rate of aeration was insufficient to keep the slices in constant motion the ascorbic acid content either failed to increase or actually decreased, depending on the tuber lot and the bubbling rate. No synthesis was found in bubbling nitrogen. Under the standard conditions, however, the ascorbic acid content of slices from suitable lots of tubers increased two to three times in 48 hours (table I). During the incubation the Q_{O_2} also increased about 30 to 100%. The respiratory quotient remained close to one during the incubation, a further indication of adequate aeration. Most batches of slices

TABLE I

INCREASE IN ASCORBIC ACID CONTENT OF POTATO SLICES FROM VARIOUS LOTS OF TUBERS DURING INCUBATION. VALUES ARE GIVEN IN MICROGRAMS PER 20 SLICES AS DETERMINED BY THE INDOPHENOL METHOD.

Tuber lots and experiments	Incubation time in hours			
	0	24	48	72
B-12-5-44	28	33	50	
B-12-13-44	34.5	79	83	
B-1-31-45	29	50	63	79
B-2-8-45	29	48.1	66	
B-2-21-45	22		48.5	49
B-3-19-45	19		55	
C-5-2-45	29.5		62.5	
E-7-1-46	64.5		175	
E-7-8-46	83		164	

showed a slight browning, as reported by STEWARD and PRESTON (**26**), except in the presence of high concentrations of inhibitors.

The significance of this study depends, of course, on the reliability of the ascorbic acid analysis. While both methods are subject to some interference, by a few other reducing compounds in one case and keto compounds in the other, it was felt that a combination of the two methods provided the most reliable characterization of the increase in ascorbic acid which was applicable to routine assay. Table II gives comparative analyses on slices before and after incubation and in the presence of iodoacetate. The differences between methods were seldom more than would be expected from sampling error and were small compared with the inhibitor effects. Nonformaldhyde-bound reducing material was usually negligible and approached 10% of the total only with strong inhibition of synthesis. There was a significant increase in dehydroascorbic acid by both the dinitrophenylhydrazine method and the indophenol method with H_2S reduction. No significant quantity of bound ascorbic acid was found.

Attempts also were made to further characterize the increased ascorbic acid by comparison of the 2,4-dinitrophenylhydrazine condensation products from slices before and after incubation with that from pure ascorbic acid. Products isolated from both types of slices agreed in melting point, absorption spectrum, and chromatographic behavior on alumina columns with the authentic ascorbic acid derivative and with the literature reports (**18**). In some cases the increase in amount of isolated osazones after incubation paralleled the analytical data; in others, however, the yields, measured spectrophotometrically, were too low to justify any conclusion. The chief difficulty, apparently, was the tendency toward formation of mixtures of the osazones of the free acid, the lactone and the pyrazalone under the condensation conditions used. Though variable, the results in several cases showed a definite increase in ascorbic acid during incubation by isolation of the osazones.

TABLE II

COMPARISON OF ANALYTICAL METHODS ON VARIOUS SLICE TREATMENTS. VALUES ARE GIVEN IN MICROGRAMS PER 20 SLICES.

Slice treatments	Indophenol method		Dinitrophenylhydrazine method	
	Total reducing substances	Non-formaldehyde bound reducing substances	Total ascorbic acid	Dehydro ascorbic acid
E-7-1				
Before incubation	66	0	64.5	3
After incubation (water)	145	4	175	20
After incubation (0.00020 M iodoacetate)	6	0	15	8
E-7-8				
Before incubation	90		83	4.5
After incubation (water)	144	7	164	22
E-7-15				
Before incubation	85.5		72	6
After incubation (water)	165	13	165	23
E-7-22				
Before incubation	75	5	71	6
After incubation (water)	121	10	93	26
After incubation (0.00005 M iodoacetate)	78	6	79	13
After incubation (0.00010 M iodoacetate)	12	0	3.5	4

The relation between ascorbic acid synthesis and respiration was investigated by comparing the effects of several respiratory inhibitors on the two processes. Malonate inhibited synthesis and respiration only at relatively high concentrations (0.01 to 0.03 M). Results were variable and slices often showed general toxicity effects, loss of turgor and fragmentation. Inhibition of synthesis by fluoride appeared at 0.001 M and was complete at 0.005 M. The higher concentration did not affect the Q_{O_2} but inhibited $Q^N_{CO_2}$ from 30 to 50%, suggesting a relationship between ascorbic acid synthesis and the glycolytic phase of respiration.

This possibility was more extensively studied with iodoacetate which blocked synthesis at much lower concentrations. Table III summarizes the results of four typical experiments. In the range from 0.00005 to 0.00020 M iodoacetate, inhibition of synthesis increased from about 30 to 95%. Corresponding concentrations of potassium acetate gave the same results as water controls. At the two higher levels of iodoacetate not only was the normal increase blocked but the original content was not maintained, suggesting that ascorbic acid was being formed continually and broken down in the slice system. In control slices, synthesis exceeded breakdown and in treated slices the final level was higher or lower than the initial level depending on the degree of inhibition of synthesis. Comparison of respiratory measurements with ascorbic acid contents showed that the

TABLE III

THE EFFECT OF IODOACETATE ON ASCORBIC ACID SYNTHESIS AND RESPIRATORY CAPACITY. ASCORBIC ACID AND MANOMETRIC FIGURES ARE GIVEN AS PERCENTAGES OF THOSE FOR WATER CONTROL AFTER INCUBATION FOR 48 HOURS. ASCORBIC ACID FIGURES ARE FROM AVERAGES OF RESULTS OF BOTH INDOPHENOL AND DINITROPHENYLHYDRAZINE METHODS.

Exp.	Initial ascorbic acid	Iodoacetate $\times 10^5$ M	Final ascorbic acid	$Q^N_{CO_2}$	Q_{O_2}	$Q^O_{CO_2}$
C-5-2	47	5	72	110	102	
		10	51	55	104	
		20	24	32	98	
E-7-1	45	5	88	47	68	55
		10	12	15	54	54
		20	4	7	42	43
E-7-15	51	5	83	96	80	86
		10	37	27	90	85
		20	9	15	48	51
E-7-22	60	5	65	29	99	84
		10	10	18	73	71
		20	14	12	65	67

$Q^N_{CO_2}$ level was more closely correlated with synthesis than either Q_{O_2} or $Q^O_{CO_2}$. This relationship is further evidence that the glycolytic phase of respiration is involved in ascorbic acid synthesis.

Discussion

The agreement found between the analyses by the indophenol method with formaldehyde binding and the dinitrophenylhydrazine method leaves little doubt that the increased reducing material in potato tuber slices is actually ascorbic acid. The oxygen requirement observed by GUTHRIE (**11**), STEWARD and PRESTON (**26**), and PROKOSHEV (**19**) was also confirmed. In addition, the parallel inhibition of $Q^N_{CO_2}$ and ascorbic acid increase by fluoride and iodoacetate has indicated that some phase of glycolysis is involved in the synthetic process.

These two observations can be interpreted in terms of a mechanism of

ascorbic acid synthesis suggested in part by SMYTHE and KING (**23**). Though the glycolytic mechanism in potato tuber tissue has not been fully established, recent evidence (**2**) strongly indicates that the classical Embden-Meyerhof pathway of yeast and muscle is followed. The relation of this pathway to the proposed scheme of ascorbic acid synthesis is diagrammed in figure 1. In the first stage of glycolysis, fructose-diphosphate is formed from starch or perhaps from sucrose or glucose. It is then split to the two trioses by the aldolase reaction. Normally these trioses are metabolized

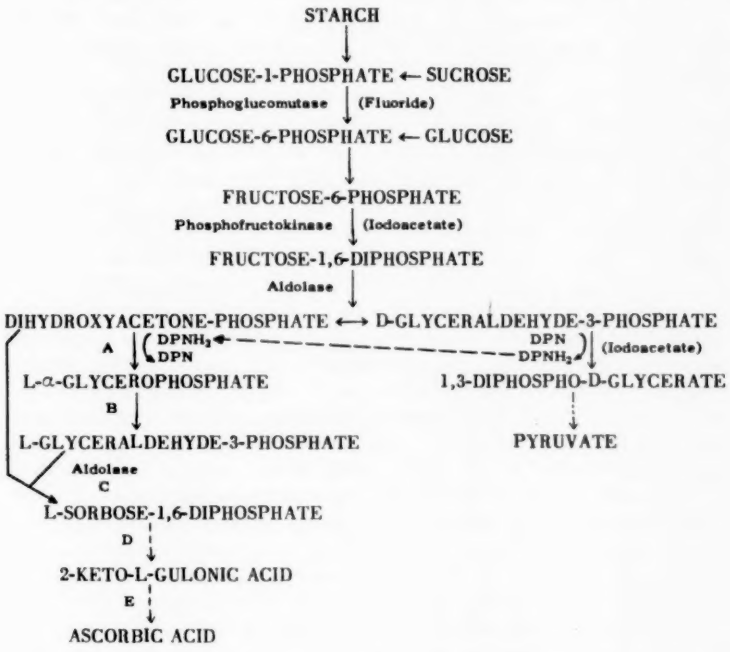

FIG. 1. The pathway of glycolysis and a proposed scheme of ascorbic acid formation.

by the diphosphopyridine nucleotide-linked oxidation of D-glyceraldehyde-3-phosphate leading to pyruvate which may then be decarboxylated aerobically or anaerobically. The first step in ascorbic acid synthesis (A, in fig. 1) would be the diphosphopyridine nucleotide-linked reduction of dihydroxyacetone-phosphate to L-glycerophosphate. This is the basic reaction of glycerol fermentation and the dehydrogenase catalyzing it has been found in tissues of higher plants (**28**). The next step (B) would be the cytochrome-linked oxidation to L-glyceraldehyde-3-phosphate. The enzyme catalyzing this reaction has been found in animal tissues (**10**) but apparently has not been studied in plants. The condensation of L-glycer-

aldehyde and dihydroxyacetone-phosphate has been carried out with aldolase preparations by MEYERHOF *et al.* (**17**). The resulting L-sorbose-phosphate would have the required L-gulose configuration of ascorbic acid. Since aldolase occurs in potato tubers (**2**) this reaction (C) would be possible. The remaining steps, oxidation of L-sorbose or its phosphorylated derivative to ascorbic acid (D and E), can be readily carried out chemically but a corresponding enzymatic mechanism has not yet been established. However, SZTARECZKY (**27**) has claimed that this conversion occurs in rat intestine. BERNHAUER (**3**) also has reported evidence of sorbose to 2-keto-L-gulonic acid conversion by *Aspergillus gluconicum* and GALLI (**8**) has shown that *Aspergillus niger* extracts will convert 2-keto-L-gulonic to ascorbic acid.

Ascorbic acid synthesis thus would depend on a source of reduced diphosphopyridine nucleotide which could be supplied by the coupled reduction of dihydroxyacetone-phosphate and oxidation of D-glyceraldehyde-phosphate as occurs in glycerol fermentation. The latter reaction usually has been consid red the site of iodoacetate inhibition of glycolysis (**9**) though COLOWICK (**5**) has recently stated that phosphofructokinase is also inhibited. Either site of inhibition could interfere with ascorbic synthesis. Fluoride inhibits several reactions involving phosphate transfer (**4, 9**) which could effect ascorbic acid formation; *e.g.*, the phosphoglucomutase reaction (**5**) and the phosphorylation of cozymase (**9**). Oxygen would be required for the L-glyceraldehyde-phosphate oxidation and possibly for the sorbose-phosphate oxidation. Ascorbic acid synthesis by this scheme would constitute a by-pass of normal glycolysis at the triose stage involving both aerobic and anaerobic reactions. The rate of synthesis, therefore, would depend both on the activity of the initial stages of glycolysis and the extent to which the L-triose by-pass competed with the normal pathway.

This scheme, though speculative, is supported by the present results with potato slices and, with one exception, is based on known biological reactions. In the latter respect it is preferable to the recent proposal of HOUGH and JONES (**12**). KING and his associates, on the basis of tracer studies with rats, preferred in 1950 (**13**) a direct conversion of glucose to ascorbic acid without splitting. However, in a more recent report (**14**) they again favor a splitting-recombination mechanism. It now appears that the majority of evidence supports a mechanism such as that proposed above which has the advantage of being based largely on well-known biological reactions. Such a mechanism, at least, provides a useful working basis for further investigation of the mechanism of ascorbic acid formation in plants.

Summary

Slices from selected lots of potato tubers increased in ascorbic acid content by two to three times in 48 hours when incubated in properly aerated aqueous media. The ascorbic acid was determined both by the indophenol method with formaldehyde binding and the 2,4-dinitrophenylhydrazine method. No significant amount of bound ascorbic acid was found but there

was some increase in dehydroascorbic acid during incubation. Fluoride and iodoacetate caused parallel inhibition of ascorbic acid synthesis and anaerobic carbon-dioxide evolution ($Q^{N}_{CO_2}$) while oxygen consumption (Q_{O_2}) and aerobic carbon dioxide evolution ($Q^{O}_{CO_2}$) were less sensitive. These facts indicate a relationship between glycolysis and ascorbic synthesis for which a proposed metabolic scheme is given.

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PREMATURE BLOSSOMING: EFFECTS OF VERNALIZATION, SEEDLING AGE AND ENVIRONMENT ON SUBSEQUENT GROWTH AND FLOWERING OF TRANSPLANTED TOBACCO

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(WITH ONE FIGURE)

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Tobacco is grown as a crop in all the coastal states from Florida to Maryland and even into New England (1). Seed is sowed in outdoor beds, and the seedlings, five to six inches tall, are set out in the field about three months later. Details of seed-bed operation are much the same throughout these sections. The beds are heavily fertilized (one or more pounds per square yard) and sowed to obtain a stand of about 20,000 seedlings per 100 square yards with less than eight square inches of soil surface per seedling. Tobacco cloth is stretched over the tops of the beds to conserve heat and moisture. The common cloth used (28×32 mesh) admits about two thirds of the light and to some extent retards heat radiation and air movement. Seedlings for transplanting are pulled from the beds preferably after watering thoroughly to soften the soil. Tobacco thus grown in seed-beds, and then in the field requires 140 to 170 days from sowing to flowering.

It is not very unusual for growers of flue-cured tobacco to find that seedlings, when set out in the field, cease vegetative growth and proceed to flower. This situation can sometimes be remedied by pinching off the terminal bud and permitting a single axillary shoot to develop. This process is costly and time consuming when premature blossoming occurs on a large scale. Many investigators have concerned themselves with this phenomenon although results obtained, generally, have not been published. Premature blossoming is usually more severe in the Southern states.

Probably the greatest variation in environmental conditions of the seed-beds throughout these regions is in illumination. Beds are sowed about January 1 in Georgia, and the seedlings are set out in the field about April 1. The corresponding dates for Maryland are about March 1 and June 1. In the intervening states the dates are intermediate. At Tifton, Georgia, the day length at sowing is 10 hours and 9 minutes, and 12 hours and 32 minutes at transplanting. In Maryland, the corresponding day lengths are 11 hours and 21 minutes and 14 hours and 44 minutes; however most varieties of tobacco are day-neutral. On the other hand, nothing is known concerning the influence of the heavy shade and dense stand on the subsequent development of the tobacco plants nor of the effects of soil moisture and of temperature. It can be assumed that the combination of short days, cloth shade, heavy stand, and cloudiness at the bed stage result in a low carbohydrate level in the seedlings.

The present study was undertaken to obtain an understanding of the factors involved in premature blossoming. To do so, it was first found necessary to devise some means for duplicating this phenomenon in the greenhouse. While environmental controls in the greenhouse were necessarily crude and tests with only a limited number of replicates could be managed, the differences and trends found were ample to determine the action of the factors studied. The many repetitions also helped to increase reliability of the data. The factors tested included vernalization of seed, age of seedling, temperature, soil moisture, and season.

Methods

Two flue-cured varieties of tobacco were studied in these experiments. During the winter of 1949 only the Gold Dollar variety was grown, but in the following year the 402 variety was included. Seeds were sowed in one-gallon glazed crocks when transplantation of seedlings after pulling was intended. Where records of leaf counts of individual plants were kept, the seeds were sowed in flats and transplanted to two-inch thumb-pots. All seedlings were transplanted from crocks or thumb-pots directly to seven-inch pots. Limitations in facilities permitted use of only six plants per lot.

Three greenhouse sections were used. Two warm sections were maintained at approximately 75° F both day and night. The other, or cool section, was kept at about 50 to 60° F at night and 65 to 75° F during the day. Soil moisture was regulated only approximately by keeping the soil somewhat moist or somewhat dry. Since tobacco is severely checked by water-logging of the soil, the tendency was to avoid over-watering of the plants kept at high soil moisture. On the other hand, daily superficial watering of the plants under low soil moisture conditions would confine most of the root growth to the upper few inches of soil. These plants were, therefore, watered sufficiently for the water to penetrate to the bottom of the pots once every three or four days. Repetition of experiments during the entire year permitted some estimation of the effects of season on premature blossoming.

All seedlings after transplanting to seven-inch pots were placed about 15 inches apart on the benches in the warm sections. Fertilizer (4–8–12) was added every 7 to 10 days to all seedlings and plants with 0.6 gm. being added to each thumb-pot and with 5 gm. being added to each seven-inch pot and crock.

Records were kept in all cases on dates of sowing, transplanting, and blossoming, also on height at blossoming. In some of the experiments, records were also kept of the total number of nodes formed following germination.

Results

Vernalization of seeds in the seed-bed by cold weather did not seem to be a factor in premature blossoming according to the data of table I. Each value is the average for 12 plants. Vernalization of all seed was begun on

TABLE I

RESPONSE OF GOLD DOLLAR TOBACCO TO VERNALIZATION OF SEEDS.

Vernalization temperature	Duration of vernalization								
	One half month			One month			Two months		
	Date of flowering	Days for flowering	Height at flowering	Date of flowering	Days for flowering	Height at flowering	Date of flowering	Days of flowering	Height at flowering
			in.			*in.*			*in.*
Control	February 24	105.3	32.1	March 10	104.6	41.3	April 2	96.2	36.0
1 to 2° C	February 23	104.4	37.8	March 12	105.1	45.9	April 1	94.5	34.7
5° C	February 23	104.6	37.8	March 9	102.9	43.5	April 1	95.8	33.2
Variation in %		±0.5	±10.6		±1.3	±5.3		±1.1	±4.0

October 27. Seeds that had started to germinate were kept in moist soil at 1 to 2° C and at 5° C for one half, one, and two months beginning on October 27. The number of days required for blossoming and the heights at blossoming with vernalized seeds were no different from those obtained with unvernalized seeds.

The seeds vernalized for one half month were brought into the warm greenhouse November 11, 1949, to permit sprouting; those vernalized for one month were brought in November 27; and those vernalized for two

TABLE II

EFFECT OF PRIOR SOIL MOISTURE AND AGE AT TRANSPLANTING OF SEEDLINGS GROWN AT 75° F ON THE SUBSEQUENT GROWTH AND FLOWERING OF GOLD DOLLAR TOBACCO AT 75° F.

Days between sowing and transplanting	Average of low and high soil moisture lots	
	Days for flowering after transplanting with variation between moist and dry series	Height at flowering with variation
		in.
43	58 ± 1	36 ± 1
50	52 ± 2	41 ± 4
57	47 ± 2	33 ± 1
64	43 ± 0	32 ± 0
71	41 ± 1	30 ± 0
78	37 ± 1	30 ± 2
85	35 ± 0	26 ± 1
92	32 ± 2	25 ± 3
99	37 ± 2	24 ± 2
106	35 ± 0	24 ± 1
113	39 ± 2	26 ± 1
120	43 ± 4	25 ± 1
127	37 ± 2	26 ± 3
134	48 ± 5	29 ± 1
141	53 ± 5	30 ± 0

months were brought in December 27. Late spring conditions after transplanting, it will be noticed, tended to decrease the number of days required from sowing to flowering in the lot vernalized two months from an average of 104.8 days to 95.5 days.

When, however, seeds were germinated and the seedlings grown in the same container and transplanted to pots at intervals, an acceleration in blossoming accompanied by retarded growth took place. Examination of table II will disclose that blossoming in the post-transplanting period was first accelerated with age of seedling but later retarded. Height at time of flowering first diminished and then increased with age of the transplanted

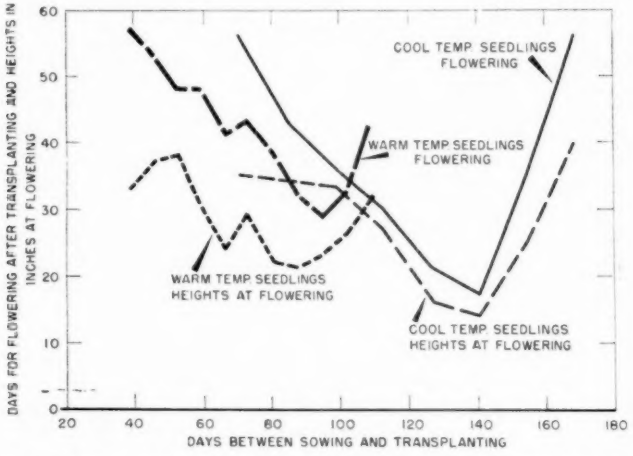

FIG. 1. Effect of seedling age and growth temperature on blossoming and height at blossoming of Gold Dollar tobacco after transfer to a warm section on transplanting.

seedlings. Table II gives the average values for two lots of seedlings sowed on November 7, 1949. Each value is the average of 12 plants, half of which were kept at low soil moisture and half at high soil moisture before transplanting. The values for each of the two series were obtained separately and then averaged. One lot was kept with soil moisture at a minimum, the other with the soil always moist. The averages for six plants of each lot were compared, and the soil moisture in the seedling stage was found to be of relatively minor influence as a factor in premature blossoming; therefore only the combined average values and their variations are given.

In experiment 2, the minimum number of days for flowering of Gold Dollar was 43 plus 58 or 101 days. In the last lot, it was 141 plus 53 or 194 days. Flowering was first accelerated, *i.e.*, premature, and later retarded if only the post-transplanting period be considered. The duration of the

seedling stage cannot be ignored, however, and if this be taken into account, it is evident that physiologically the basic phenomenon is one of variation in delay of flowering. The number of days from sowing to flowering increased in each lot of successive transplants. Maximum reduction in time for flowering in the post-transplanting period was 44.8%, and the maximum reduction in height was 33.3%.

The next factor to be checked was temperature. Seeds were sowed and placed both in warm and cool sections of the greenhouse. The seedlings

TABLE III

EFFECT OF AGE OF TRANSPLANTED SEEDLINGS ON TIME FOR FLOWERING, HEIGHT AT FLOWERING, AND LEAVES FORMED WHEN GROWN AT LOW AND HIGH TEMPERATURES.

Variety	Days between sowing and transplanting	Seedlings grown at cool temperature			Seedlings grown at high temperature		
		Days for flowering after transplanting	Height at flowering	Number of nodes at flowering	Days for flowering after transplanting	Height at flowering	Number of nodes at flowering
			in.			*in.*	
Gold Dollar	57	41	26	22	44	28	22
	71	49	23	26	50	19	25
	85	36	24	23	41	28	26
	99	32	15	21	34	21	25
	113	29	17	21	27	18	26
	127	23	12	22	27	15	26
	141	18	8	22	24	14	29
	155	19	10	21	32	17	29
402 variety	57	44	33	23	54	40	24
	71	47	30	25	47	31	24
	85	37	22	22	45	29	27
	99	41	23	23	48	27	29
	113	29	13	21	44	27	30
	127	31	14	22	46	26	31
	141	23	11	22	32	19	28
	155	20	11	19	32	22	32

were transplanted to seven-inch pots and grown in the warm sections. A definite indication of a temperature effect appeared in both the Gold Dollar and 402 varieties. Accelerations in blossoming and inhibitions in height were definitely greater with seedlings grown at the lower temperature.

The type of response and its intensification by low temperatures is illustrated in figure 1 showing the data of an experiment with Gold Dollar. Both lots of seeds were sowed on October 24. It will be noted that with increasing age of transplanted seedlings, blossoming was first accelerated and height at blossoming diminished. These changes were reversed with still greater age of transplanted seedlings and gradually disappeared. Low temperatures during the seedling stage caused a general lag in growth and

development of the plants and an intensification in premature blossoming as well.

The data of another experiment are presented in table III. The seeds were sowed October 16, 1950 and grown in the warm greenhouse section after transplanting. Here the accelerative effect of seedling growth at low temperature is noticeable with the plants of the Gold Dollar variety transplanted at 85 and 99 days. With the 402 variety, the acceleration was evident with plants transplanted at 85 to 155 days. A record was kept in this experiment of every node formed by the seedlings from the cotyledon stage

TABLE IV

EFFECT OF AGE OF TRANSPLANTED SEEDLINGS GROWN AT 75° F ON TIME FOR FLOWERING, HEIGHT AT FLOWERING, AND LEAVES FORMED AT 75° F.

Variety	Days between sowing and transplanting	Leaves formed when transplanted	Days for flowering after transplanting	Height at flowering	Nodes at flowering
				in.	
Gold Dollar	36	6	66	37	33
	50	9	58	44	35
	64	11	56	34	34
	78	12	45	33	31
	92	15	36	29	29
	106	16	38	26	28
	120	17	34	23	29
	134	20	31	22	28
	148	22	9	15	29
	149	29	0		
402 variety	36	6	66	40	38
	50	9	64	40	33
	64	11	61	39	34
	78	12	50	40	32
	92	15	45	33	33
	106	16	46	33	31
	120	16	45	28	28
	134	17	40	28	28
	148	18	29	20	27
	162	21	22	14	27
	163	29	0		

onward by notching the leaves from the time the seedlings were transplanted from the seed flats to two-inch thumb-pots until they blossomed. The effect of age of seedling at transplanting on the number of nodes formed was definite. The number of nodes formed in both varieties tended to increase with the age of the seedling transplanted for those started in the warm section. The average number of nodes formed before blossoms opened was lower for seedlings started at low temperatures than for those started at 75° F. The average number of nodes per plant of the Gold Dollar variety was 22.25 for seedlings grown at the low temperature and 27.25 at high temperature, or an 18.3% increase. For the 402 variety, the values were 22.125 and 28.125, or a 21.3% increase.

The maximum reduction in time for blossoming with Gold Dollar in experiment 3 was 56.1%, and the maximum reduction in height was 69.2%. The corresponding values for the 402 variety were 54.5 and 66.7%. The plants transplanted earliest again blossomed in a minimal number of days from sowing.

The effect of warm temperatures during the seedling stage on the number of nodes at flowering was not always the same. In table IV, the number of nodes diminished with age at transplanting in both varieties of tobacco as did the time for blossoming and the height at blossoming.

This difference in numbers of nodes at flowering associated with the age of seedling started at warm temperatures appeared to be dependent upon the season, the only uncontrolled factor. The results in table III were obtained with seed sowed on October 16, the experiment lasting 155 plus 32 days. Those in table IV were obtained with seeds sowed on July 10, and the experiment lasted 162 plus 22 days. That is to say, in the former experiment the seedlings and plants were grown from winter to summer, and in experiment 4, from summer to winter. Maximum reductions in time for flowering and heights were 86.4% and 60.0%, respectively, for Gold Dollar and 66.7% and 65.0% for the 402 variety. The minimal number of days from sowing to flowering again occurred only in the earliest plantings.

Discussion

An examination of these data will reveal that the designation of "premature blossoming" is misleading. The data deal with variations in a retardation in flowering by day-neutral seedlings induced in the seed-bed. This will become clear when it is realized that in the greenhouse the average time for earliest blossoming is 95 days for Gold Dollar and 100 days for the 402 variety. The minimum number of days for individual flowering encountered during these experiments was 83 days for Gold Dollar and 87 days for the 402 variety. Since the duration of growth in the seed-bed alone usually ranges from 75 to 90 days, flowering could, under optimum conditions, occur shortly after transplanting to the field. The fact that flowering does not generally take place would indicate that environmental factors in the seed-bed introduce a lag in the ripening process of the seedlings in which about 140 to 170 days are required from sowing to flowering. This interpretation is substantiated by the fact that most rapid flowering in each trial occurred almost without exception in the earliest transplantings in the greenhouse.

Results similar to those tabulated were obtained in other experiments that were performed. Increasing age of seedling when transplanted resulted in a decrease in the subsequent time required for blossoming and in the height at blossoming. If transplantings were continued long enough, however, these responses were reversed. Blossoming again took longer and the plants were taller at time of blossoming. These responses would indicate that growth in height, total number of leaves or internodes formed, and blossoming can proceed at different rates and are each independently subject to modification by environmental conditions.

The age of the seedling when it was transplanted seemed to be the major factor in these results, whereas soil moisture in the seedling stage seemed to have little or no influence. Low temperatures during the seedling stage resulted in a marked increase in response. It is also probable that other factors may be concerned. The individual values for each of six plants in a lot often differed widely with apparently identical treatments. The explanation may consist to some extent in the lack of genetic uniformity of the varieties employed.

Attention is called to the fact that the low temperatures employed here are not as extreme as those often encountered in the seed-beds. It can be expected that the grower will sometimes experience premature blossoming in a more intensified form than that found in these experiments. Prevention of premature blossoming of tobacco would be aided, therefore, by the later sowing of seed and the use of higher temperatures during the seedling stage. This procedure would have the effect of accelerating growth and thereby decreasing the age of seedlings when they are of a size suitable for transplanting. Pre- and post-transplanting soil moisture could not be determined to have a definite influence on premature blossoming.

The use of higher seed-bed temperatures may also be desirable because of a significant increase indicated in the number of leaves formed per plant. This increase was about 20% for both varieties in experiment 3. It would be advisable, therefore, to obtain data on the relation of seed-bed temperature to number and weight of leaves harvested per plant to determine just what proportion of this potential increase could be realized in practice.

Summary

The phenomenon of premature blossoming (arrested vegetative growth and concomitant flowering) of tobacco when transplanted from seed-bed to field was duplicated under greenhouse conditions. A study was made on the effect of seed vernalization and environmental growth conditions before transplanting of seedlings on the subsequent time required for flowering, height at flowering, and total number of leaves formed. The flue-cured varieties, Gold Dollar and 402, were used. The minimal number of days from sowing to flowering in the greenhouse was 83 days for the Gold Dollar and 87 days for the 402 variety. These values were approached only when seedlings of minimal age were transplanted. Plants grown in the field exhibited retarded development since they required 140 to 170 days from sowing to flowering. With increasing age of seedlings used for transplanting, the subsequent time required for flowering, height, and number of nodes at flowering decreased at first but later increased. In experiments continuing into the summer, these trends were minimized or even reversed (height and leaves formed before flowering). Low temperatures during the seedling stage intensified these trends. Vernalization of seed and soil moisture content had no effect. Premature blossoming in field tobacco, it is surmised, could be minimized by the use of higher temperatures and the later sowing

of seed, both tending to decrease age of seedlings when of a size suitable for transplanting. High temperatures during the seedling stage also increased average number of leaves formed per plant by about 20% with both varieties.

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COMPOSITIONAL AND PHYSIOLOGICAL CHANGES ASSOCIATED WITH THE CHEMICAL DEFOLIATION OF COTTON[1]

WAYNE C. HALL AND HARRY C. LANE

(WITH THREE FIGURES)

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In a previous publication (**13**) evidence was presented in support of the auxin–ethylene balance hypothesis as an explanation for natural leaf abscission. It was found that the two commercial defoliants, Shed-A-Leaf (sodium chlorate–pentaborate) and Endothal (disodium-3,6-endoxohexahydrophalate + ammonium sulphate), accelerated ethylene production in cotton leaves. This observation has since been confirmed for other defoliants by another investigator (**16**).

The present study was designed to investigate the role of ethylene in natural and chemically induced leaf abscission. It has also become of interest to re-investigate the role of the carbohydrate and nitrogen fractions of the leaf in relation to defoliation as well as to determine any physiological or compositional changes in the cotton leaf blade and plant during chemically induced defoliation and, if possible, to assess their importance to the basic mechanism of abscission. The first problem has been the subject of two recent papers (**5, 9**). Evidence has been presented by V. L. HALL (**9**) that the susceptibility of the cotton plant to defoliation varies inversely with reducing sugars, starch, and dextrin in the leaves, whereas his data showed a direct relation between total nitrogen in the leaf and per cent. leaf drop. On the other hand, EATON and ERGLE (**5**) have provided extensive data showing that the percentage of bolls shed by cotton plants is not directly related to the carbohydrate and nitrogen status of the plant.

DENNY (**6**) has reviewed the literature up to 1933 concerning compositional changes in leaves prior to leaf fall. Although the bulk of the earlier workers reported an autumnal shift in leaf composition, he (**6**) concluded from his own work with *Viburnum dentatum* and *Syringa vulgaris* that the dry weight of leaves was nearly constant for the period of sampling (41 days prior to frost); no important changes were observed in sugars or polysaccharides, while nitrogen losses were confirmed only for Viburnum. Abscission records were not kept, and therefore it is not known whether any correlation actually existed between leaf drop and leaf composition.

BROWN and ADDICOTT (**3**) have reported upon the anatomy of leaflet abscission in *Phaseolus vulgaris* and have noted that starch disappeared rapidly from all tissues except those in the abscission zone, and that there was a polar movement of sucrose when applied to the pulvinal region. The sucrose polymerized to starch in the stem. To the authors' knowledge, how-

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ever, a quantitative study of compositional changes occurring during chemical defoliation has not been reported heretofore.

Methods

1950 Experiments

Four experiments were conducted during the spring, summer, and fall of 1950, with the experiment conducted during the fall continuing into late winter and early spring of 1951. The experiments of spring, summer, fall, and late winter–early spring will be referred to as experiments 1, 2, 3, and 4. Stoneville 2B, Deltapine, Acala, and Rowden 41B varieties were used in the first three experiments, whereas only the Stoneville 2B variety was used in the fourth experiment. All plants were started in the greenhouse and grown in three-gallon jars. Experiment 1 was started in early spring and grown in Lufkin fine sandy loam which is a poor soil low in nitrogen. The defoliants were applied on August 15. The plants of experiment 2 were grown simultaneously with those of experiment 1 and differed only in that they were cultured in manured Houston clay with two applications of ammonium nitrate. They were sprayed on August 23. The plants of experiment 3 were identical to those of experiment 2 except floral buds were removed daily. Experiment 4 was started in the greenhouse during the winter of 1950 and was terminated in the spring of 1951. The cultural conditions for the experiment were the same as for experiment 2. Experiment 4 was designed to study the influence of maleic hydrazide and sucrose, singly and in combination with Endothal and Shed-A-Leaf.

Eighty plants were used in both experiments 1 and 2 (20 of each variety); 24 plants were used in experiment 3 (six of each variety); and 80 plants were used in experiment 4. All plants were defoliated when approximately 50% of the bolls had opened. A commercial wetting agent, Nonic 218, was added to all defoliants which were freshly prepared just prior to application. The defoliant sprays were applied with a power-driven atomizer sprayer until the foliage was wetted. A high defoliant concentration of 5% was utilized in all experiments except experiment 4 where a 2% concentration was employed. Sucrose was applied at 3.5 and 5% and maleic hydrazide at 4800 p.p.m. (table I).

In all experiments, the number of leaves per plant was determined prior to defoliant application and seven to nine days after application. From the difference the percentage defoliation was determined. This figure included both the leaves that abscised naturally and those that fell when gentle pressure was applied to simulate field conditions. All data presented are the averages of the individual treatments and, in the case of the first three experiments, represent a composite of the four varieties used.

1951 Experiments

Two lots of Stoneville 2B plants were grown in manured Houston Clay in the greenhouse at College Station. The first lot involved 10 plants which

were defoliated on June 15 with Shed-A-Leaf at a rate equivalent to six pounds per acre. The second lot of 24 plants provided the plant material for both the ethylene experiment (tables II and III) and the respiration measurements (table IV). The plants defoliated with 100 p.p.m. ethylene were cut off at the ground line and placed in beakers of water under large bell jars with the gas for 72 hours. Leaf samples were taken at the beginning, at 48 hours, and at 72 hours. Comparable controls were also placed under bell jars for 48 hours to see what effect the bell jar environment had upon defoliation and leaf composition. The plants used for the respiration measurements were sprayed with 2% Shed-A-Leaf, and leaf samples were taken three hours later and at 24 hour intervals up to 72 hours.

Replicated field plot experiments were conducted at the Weslaco, Temple, and Lubbock stations during the summer of 1951. These stations were selected because they represent diverse farming practices, climates, and growing conditions. Therefore it is believed that some significance can be attached to similarities in plant reaction to chemically induced defoliation when grown under such greatly different conditions, whereas marked deviations from the norm are of questionable significance. A uniform defoliant (Shed-A-Leaf), at the rate of seven to eight pounds per acre, was applied by means of tractor-mounted spray equipment. The mode of action of Shed-A-Leaf was compared to that of ethylene used at College Station (tables II and III).

Leaf samples were collected at the time of defoliant application at all locations. At College Station, samples were also taken 24, 48, and 72 hours after application. In the Lower Rio Grande Valley and Temple experiments, leaf samples were collected 24 and 48 hours after application, while at Lubbock, samples were collected 48 and 72 hours after application (table III). Daily observations were made of plants at all localities throughout most of the defoliation process. Defoliation counts were made as soon as the abscission process was completed, and the per cent. of leaf fall for the plots was averaged. With the exception of the plants at Lubbock, the per cent. of leaf fall was determined about eight days after the defoliant was applied.

The experiment in Lower Rio Grande Valley was performed near Donna, Texas, under irrigated conditions of ample moisture. The Deltapine variety of cotton was used and the defoliant was applied in early July. The Temple experiments utilized the Empire variety of cotton grown under extremely unfavorable conditions. At the time of defoliant application (August 20), the plants were under very serious drought stress. The plants at Lubbock were grown under what may be called semi-dry land conditions. Approximately 11 inches of rain fell during the season, which was supplemented by a four acre-inch irrigation in April and a two acre-inch irrigation in late July. Application of the defoliant to the Storm Proof cotton variety normally grown in the High Plains was made in early October.

Sampling, analytical and chemical methods

In 1950, the leaves on the main stalk of the control plants of the four experiments were sampled at the time of defoliant application, those of the plants of experiment 4 sprayed with 5% sucrose were sampled at 3 and 55 hours after application, and those of the plants sprayed with maleic hydrazide were sampled one week after application. In 1951 the plants were sampled as outlined in the previous section and summarized in tables II and III and figures 1 and 2. The plants at College Station were separated into leaf blades, petioles, and stems (figs. 1, 2). Only leaf blades were sampled at the other stations. The stem portion included the upper three fourths of the main stalk; the woody basal portion was discarded. In all

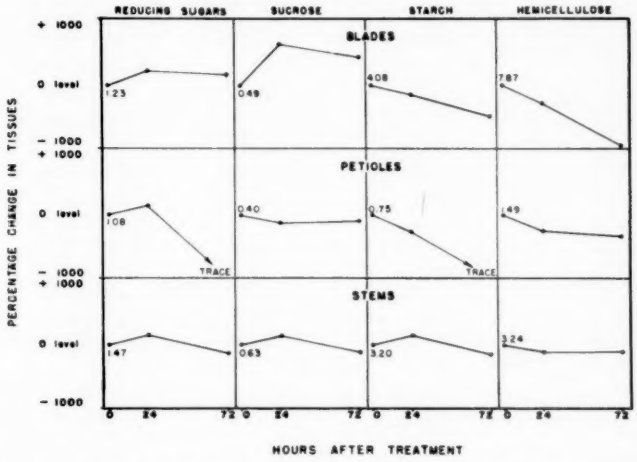

FIG. 1. Changes in carbohydrate fractions of blades, petioles, and stems during defoliation of cotton plants grown at College Station in 1951. All values are expressed as per cent. of dry weight.

cases the samples were oven-dried as soon as possible at 80° C and ground to pass an 80-mesh screen. Carbohydrates were determined by methods described in detail elsewhere (**4, 11**). Total nitrogen, including nitrates and nitrites, was determined by the modified Kjeldahl method using salicylic acid and sodium thiosulphate. Soluble nitrogen, extracted with 80% ethanol from oven-dry material, was determined by the Kjeldahl method after preliminary reduction with reduced iron powder.

The respiratory rates of control leaves and leaves sprayed with Shed-A-Leaf were measured in a standard Warburg respirometer by the leaf-disk method previously described (**11**). Oxygen uptake was measured with 0.2 ml. of 20% KOH in the center well to absorb CO_2. Carbon dioxide was determined by the direct method of Warburg. All measurements were made

in very diffuse light at 30° C, and at a stroker rate of 120 strokes per minute. Fifteen minutes of equilibration were allowed before closing the manometer stopcocks. Gas exchange was measured for one hour in all cases. Several flasks of the same treatment were used, and the two results in closest agreement were averaged for the data of table IV.

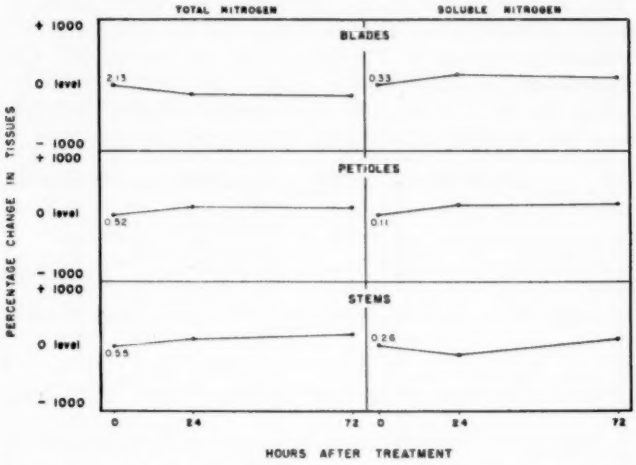

FIG. 2. Changes in nitrogen fractions of blades, petioles, and stems during defoliation of cotton plants grown at College Station in 1951. All values are expressed as per cent. of dry weight.

Results

1950 EXPERIMENTS

Five different techniques were utilized in an attempt to alter the carbohydrate and nitrogen composition of the cotton plant: (a) The plants were grown in a low nitrogen supply, a procedure which has been shown to cause carbohydrate accumulation as well as decreased nitrogen content (**4**); (b) The plants were grown on a high nitrogen supply causing an increase in nitrogen content and a decrease in carbohydrate content; (c) Sucrose was applied as a spray to induce a build up in carbohydrate content; (d) The flower buds were removed which caused an increase in carbohydrate content as has been shown not only for cotton (**4**) but for other plants as well (**1, 10**); (e) Maleic hydrazide, which has been reported to cause an accumulation of carbohydrates in the cotton leaf (**18**), was applied as a spray.

The effects of these treatments on the carbohydrate composition of the main stalk leaves and their influence upon the per cent. of defoliation are summarized in table I. It was found that the low nitrogen treatment in experiment 1 increased the carbohydrate content when compared to the plants supplied with high nitrogen in experiment 2. The debudding and

TABLE I

THE RELATIONSHIP OF CARBOHYDRATE COMPOSITION (AS PER CENT. DRY WEIGHT OF MAIN STALK LEAVES) AND AVERAGE PERCENTAGE DEFOLIATION AS SHOWN IN EXPERIMENTS OF 1950.

Experiment and treatment	Average per cent. defoliation	Carbohydrates of main stalk leaves at time of spraying				
		Reducing sugars	Sucrose	Starch	Hydrolyzable residue	Total
Experiment 1, low N supply						
Control	00.0	1.03	1.03	2.90	7.25	12.48
5% Endothal	12.7					
5% Endothal + 3.5% sucrose	76.7					
5% Shed-A-Leaf	14.5					
5% Shed-A-Leaf + 3.5% sucrose	60.5					
Experiment 2, high N supply						
Control	00.0	0.70	0.91	2.30	6.20	10.11
5% Endothal	26.5					
5% Endothal + 3.5% sucrose	32.3					
5% Shed A-Leaf	26.5					
5% Shed-A-Leaf + 3.5% sucrose	57.5					
Experiment 3, debudded						
Control	00.0	0.93	1.32	2.95	7.80	13.00
5% Endothal	85.5					
5% Shed-A-Leaf	82.5					
Experiment 4, maleic hydrazide, sucrose						
Control	00.0	0.86	2.02	2.35	7.40	12.63
5% sucrose (3 hrs. after application)	00.0	1.19	2.62	2.07	7.10	12.98
5% sucrose (55 hrs. after application)	00.0	0.86	2.05	3.32	7.20	13.43
4800 p.p.m. maleic hydrazide (1 week after application)	00.0	1.88	1.20	4.80	7.90	15.60
2% Endothal	42.5					
2% Endothal + 5% sucrose	94.3					
2% Endothal + 4800 p.p.m. maleic hydrazide	66.8					
2% Shed-A-Leaf	83.6					
2% Shed-A-Leaf + 5% sucrose	94.4					
2% Shed-A-Leaf + 4800 p.p.m. maleic hydrazide	89.2					

maleic hydrazide treatments caused the greatest increase in leaf carbohydrates; whereas some increase in leaf carbohydrates resulted from the use of the sucrose sprays.

It is recognized that the defoliant concentration employed in the first three experiments was too high for optimum results, but this concentration was employed to ascertain the possible tonic or protective effect of sucrose in alleviating defoliant toxicity. The poor defoliation obtained with Endothal or Shed-A-Leaf alone in experiments 1 and 2 was at least partially attributable to the high defoliant concentration. Little evidence exists in

these data (table I) to support the report of V. L. HALL (9) that a correlation exists between leaf carbohydrates and the percentage defoliation effected. Rather, the correlation in the present data appears to be more an interaction between defoliant treatment and leaf composition during the defoliation process.

1951 EXPERIMENTS

A few notations of general interest are presented on plants defoliated with Shed-A-Leaf at the four locations in 1951 and others not included in this report. The growing conditions modify greatly the time and nature of the plant response to chemical defoliants as reported by V. L. HALL (9).

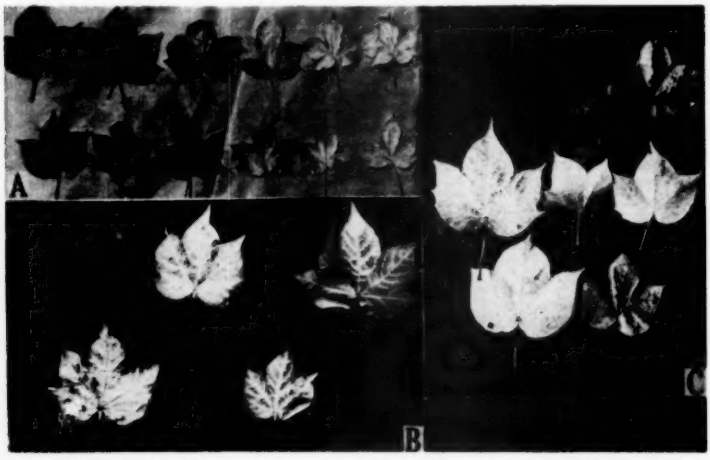

FIG. 3. Representative cotton leaves photographed during the abscission process showing the similarity in the pattern of degreening (loss of chlorophyll). A. Leaves from Lower Rio Grande Valley plants defoliated with Shed-A-Leaf. Left to right, progressive stages from defoliant application until leaf fall. B. Leaves enclosed with 100 p.p.m ethylene for 72 hours. C. Leaves from plants grown at College Station showing degreening stages during natural abscission.

Plants grown in the greenhouse in general have responded very quickly to the defoliant and exhibit signs of defoliant action a few hours after application, and defoliation is complete within five to seven days. Conversely, the plants grown in the High Plains area of Texas always respond very slowly to any type of defoliant. It generally takes several days before any visible symptoms of defoliant action on the plants can be observed, and in many cases three weeks are required before defoliation is complete. Some differences in response can be attributed to poor spray coverage, but for greenhouse plants or the small type of plant usually grown on the High Plains, complete coverage is not difficult to achieve. However in the Lower Rio Grande Valley, the rank type of growth, with 40 to 100 leaves per plant not uncommon, makes good coverage extremely difficult.

The usual leaf symptoms appearing 24 hours or longer after defoliant application are chlorotic spotting and localized dehydration of the leaf. The size of the spots is regulated by the size of the defoliant droplet and the rapidity of action of the defoliant. The spotting is probably caused by localized absorption and partial tissue destruction in the immediate area of contact with the droplet. Occasionally the leaves become red. Of particular interest was the degreening (loss of chlorophyll) that first appeared in some plants in the early stages more or less concomitantly with the spotting effect and which increased until leaf drop. In most cases, however, this chlorosis is masked in the field by the rapidity of defoliant action resulting

TABLE II

THE RELATIONSHIP OF CARBOHYDRATE AND NITROGEN COMPOSITION (AS PER CENT. DRY WEIGHT OF LEAF BLADES) AND AVERAGE PERCENTAGE DEFOLIATION AS SHOWN IN EXPERIMENTS OF 1951.

Sample	Average per cent. defoliation	Carbohydrates					Nitrogen	
		Reducing sugars	Sucrose	Starch	Hydrolyzable residue	Total	Total	Soluble
College Station Grown in 3-gal. jars outside. Defoliated with SAL. 6 lbs./acre	87	1.23	0.49	4.08	7.85	13.67	2.13	0.33
Lower Rio Grande Valley Irrigated, field grown. Defoliated with SAL. 8 lbs./acre	65	1.23	0.30	4.05	8.20	13.78	2.00	0.26
Temple Dry land, field grown. Defoliated with SAL. 8 lbs./acre	32	0.63	1.95	3.02	6.00	11.60	2.27	0.40
Lubbock Semi-dry land, field grown. Defoliated with SAL. 7 lbs./acre	79	1.05	0.56	0.39	2.18	4.18	3.35	0.49
College Station Grown in 2-gal. jars outside. Defoliated with 100 p.p.m. Ethylene gas	96	2.50	1.80	4.75	8.50	17.75	2.56	0.44

in quick drying and browning of the foliage, which soon obscures the degreening that occurs. In this connection a series of photographs of representative cotton leaves from the Lower Rio Grande Valley plants were photographed showing the progressive loss of chlorophyll from the time of defoliant application until leaf drop (fig. 3 A). These leaves are to be compared with leaves induced to abscise by treatment with ethylene gas (fig. 3 B) and leaves abscising naturally (fig. 3 C). The similarity in the pattern of degreening in all three cases is striking.

In general agreement with the 1950 results, no definite trends or correlations are apparent between the carbohydrate and nitrogen fractions of the leaf blade and the average percentage defoliation obtained (table II). The

Lubbock samples were the lowest in total carbohydrates and highest in total and soluble nitrogen but had a higher percentage of defoliation than the Temple or Lower Rio Grande Valley plants. On the other hand the College Station plants which were defoliated with 100 p.p.m. ethylene were highest in starch, reducing sugars, and total carbohydrates, intermediate in nitrogen, and gave the highest percentage defoliation. The extremely low reserve carbohydrate content of the Lubbock plants is noteworthy. It should

TABLE III

CHANGES IN CARBOHYDRATE AND NITROGEN COMPOSITION OF LEAF BLADES DURING DEFOLIATION AS SHOWN IN EXPERIMENTS OF 1951. ALL VALUES AS PER CENT. OF DRY WEIGHT.

Treatment	Time after application	Carbohydrates					Nitrogen	
		Reducing sugars	Sucrose	Starch	Hydrolyzable residue	Total	Total	Soluble
	hrs.							
				College Station				
Control	0	1.23	0.49	4.08	7.87	13.67	2.13	0.33
6 lbs. SAL.	24	2.60	3.01	3.15	2.88	11.64	1.64	0.58
6 lbs. SAL.	72	2.07	2.03	0.83	0.85	5.78	1.46	0.35
				Lower Rio Grande Valley				
Control	0	1.23	0.30	4.05	8.20	13.78	2.00	0.26
8 lbs. SAL.	24	0.75	1.01	3.41	6.15	13.32	2.41	0.30
8 lbs. SAL.	48	1.20	0.78	3.15	6.00	11.13	2.01	0.27
				Temple				
Control	0	0.63	1.95	3.02	6.00	11.60	2.27	0.40
8 lbs. SAL.	24	0.13	0.44	2.75	6.10	9.42	2.24	0.57
8 lbs. SAL.	48	0.75	1.29	1.34	6.20	9.58	2.22	0.44
				Lubbock				
Control	0	1.05	0.56	0.39	2.18	4.18	3.35	0.49
7 lbs. SAL.	48	0.83	0.49	0.20	2.53	4.06	3.07	0.56
7 lbs. SAL.	72	0.99	0.99	None	1.16	2.64	2.56	0.50
				College Station				
Control	0	2.50	1.80	4.75	8.50	17.75	2.56	0.44
Control	48	1.23	1.82	3.64	7.75	14.44	2.51	0.45
100 p.p.m. $CH_2=CH_2$	48	0.60	0.68	0.51	7.11	8.90	2.54	0.56
100 p.p.m. $CH_2=CH_2$	72	0.10	0.15	None	2.63	2.88	1.96	0.48

be pointed out also that the relatively low amount of defoliation of the Lower Rio Grande plants was in part due to inadequate coverage of the foliage.

When changes in the carbohydrate and nitrogen composition of leaf blades were followed during the early stages of defoliation (up to 72 hours) of plants grown at the different locations, several trends in common were noted. The highest percentages of defoliation (table II) were associated with a rapid depletion of carbohydrates, particularly the reserve and struc-

tural carbohydrates (starch, hemicellulose, etc.) (table III). These fractions underwent rapid hydrolysis as shown by the general increase in their equivalent in soluble sugars at 24 and 48 hours. Eventually, however, considerable translocation or metabolic transformation occurred as evidenced by a net decrease in total carbohydrates of the blades. Changes in total nitrogen and soluble nitrogen followed much the same trends. The plants showing the most defoliation were those showing the greatest change in total nitrogen during the 72 hour period.

The control plants placed under bell jars for 48 hours showed some decrease in leaf blade carbohydrates and nitrogen. However, the change in these constituents in the plants enclosed with ethylene was much greater suggesting that ethylene accelerates enzyme activity, an effect observed in other plant organs (**12, 15**). The results with ethylene, although more pro-

TABLE IV

EFFECT OF DEFOLIANT (SAL.) ON GAS EXCHANGE IN COTTON LEAF DISKS. MEASUREMENT WAS MADE THREE HOURS AFTER SPRAY APPLICATION AND AT 24 HOUR INTERVALS THEREAFTER.

Treatment	Time after application	Q_{O_2}	Per cent. of control	Q_{CO_2}	Per cent. of control	R. Q.
	hrs.					
Control	3	1.34	....	1.20	....	0.89
Defoliant	3	2.89	215.6	2.71	225.8	0.93
Control	27	1.65	....	1.63	....	0.99
Defoliant	27	3.12	189.0	2.82	173.00	0.90
Control	51	1.76	....	1.93	....	1.09
Defoliant	51	3.80	215.9	3.73	193.2	0.98
Control	75	1.70	....	1.84	....	1.08
Defoliant	75	3.45	202.8	3.56	193.4	1.03

nounced, are comparable to those obtained with the defoliant at the other stations, and suggest a common mode of action.

Analyses of leaf blades, petioles, and stems of the College Station plants (fig. 1) indicated the nature of the changes observed in the leaf blades which are summarized in table III. Chemical analyses of the samples taken at 24 hours show that the reserve fractions of the petioles and the blades were undergoing simultaneous hydrolysis. A comparison of the values for soluble carbohydrate and nitrogen fractions at 24 hours with the initial values indicates that these products were also being translocated out of the blade and petiole into the stem. The higher starch value at 24 hours in the stem compared to the initial content in this organ indicates polymerization. By 72 hours, both starch and reducing sugars in the petioles had fallen to trace amounts, although both fractions were still present in the blades. Total nitrogen in the petioles at 72 hours, however, had not changed appreciably, although soluble nitrogen had steadily increased. These changes in the

petiole probably have special significance in abscission. The decrease in total carbohydrates of the stem taking place between 24 and 72 hours suggests that translocation was occurring from the stem to the roots. Unfortunately, roots were not sampled to substantiate this interpretation.

A comparison of the various carbohydrate fractions in the leaf blades, petioles, and stems (figs. 1, 2) before and up to 72 hours after defoliant application shows a deficit when the fractions are totaled. It appeared that the difference was more than could be accounted for by translocation to the roots and therefore an accelerated respiration was indicated. This interpretation was confirmed by respiratory determination employing the Warburg apparatus (table IV). The respiratory rate was approximately doubled in the plants sprayed with defoliant during the period of 72 hours as measured either by oxygen uptake or carbon dioxide production. The respiratory quotient suggests that carbohydrates were the primary substrates being respired under aerobic conditions.

Discussion

By considering the observations and results of this study as a whole, several events assume a paramount position in the chemically induced type of abscission process which have much in common with changes observed in the natural type and ethylene-induced type of abscission. The first of these events is the degreening phenomenon that occurred in all three types of abscission. Gawadi and Avery (**8**) noted that in the abscission process of leaf fall separation of the leaf from the stem occurs when the leaves begin to lose their green color. Brown and Addicott (**3**) listed the events leading to abscission in the bean leaf and described the pulvinus as changing in color from deep green through pallid green and yellow-green to yellow. They noted a positive correlation between the color of the pulvinus and abscission. Typically, if the pulvinus is green, leaf fall will not take place; if it is yellow, only a light touch is necessary to bring about separation. Olmstead (**19**) studied the effect of photoperiod on abscission in the sugar maple and stated that abscission may involve a loss of green color but this loss may be a side effect of the aging process related to photoperiod and may not in itself lead directly to abscission. In studying the degreening of oranges, Roper and Miller (**20**) noted that the synthetic auxins offered no promise as degreening agents and none had a major effect upon respiration. On the other hand, ethylene greatly stimulated the respiratory rate and gave excellent degreening. They concluded from their work that reactions which cause degreening are natural ones and the addition of ethylene is the most effective means known to make these processes occur at the maximum rate possible. Cotton petioles were observed to redden slightly, lose green color, and have total loss of color in the blade and petiole when the plants were enclosed in an ethylene atmosphere where a high rate of abscission occurred (**13**). However, when the cotton plants were pretreated with naphthaleneacetic acid, degreening of leaves or petioles did not take place

nor did they abscise to any extent. In the present study, the similarity in the pattern of degreening noted during chemically induced abscission, natural abscission, and ethylene-induced abscission, indicates rather clearly the possibility that ethylene is the causal agent in all three cases.

Other important events, such as the rapid hydrolysis of reserve compounds to soluble constituents, their polar translocation in the plant, and the greatly stimulated respiratory rate, all seem to occupy key roles in the defoliation process. The results of this study, as well as other published (**3, 13, 17, 21**) and unpublished observations (**14**) lead us to postulate that the physiological course of events during chemically induced defoliation proceeds, more or less, along the following pathway. As the defoliant strikes the leaf, it is absorbed and brings about a temporary inhibition of the metabolic processes of the leaf; respiration is slowed for an hour or two (**13**), leaf processes are partially inactivated, and some localized tissue destruction occurs. It is believed that these reactions lower the auxin content (**13, 17**) and are followed by a rapid stimulation of both hydrolytic and respiratory enzymes. The reserve constituents of the leaf, such as starch, the hemicelluloses, and other structural carbohydrates, and the proteins and insoluble nitrogen fractions are rapidly hydrolyzed as evidenced by their decrease and by the concurrent increases in the soluble fractions, such as sugars and soluble nitrogen. Respiratory enzymes become greatly stimulated as shown by accelerated oxygen uptake and increased carbon dioxide output as soon as three hours after defoliant application. The respiratory quotient indicates that the soluble carbohydrates are the primary substrates being oxidized under aerobic conditions. In some cases it has been shown that increased aerobic respiration is favorable to increased ethylene production (**7, 12, 13, 15**). The initial evolution of ethylene may further lower the auxin level or inactivate auxin, shifting the auxin-ethylene ratio in the favor of ethylene; initiating the events leading to degreening and chlorophyll destruction (**20**) and further stimulating both hydrolase and respiratory enzymes. Bennett (**2**) has shown that the hemicelluloses of the corn stalk are made up largely of pentosans which yield pentoses upon hydrolysis. In other work (**12**) it has been demonstrated that the pentoses, particularly arabinose, served as excellent substrates for ethylene production in *in vitro* experiments. With the high rate of hydrolysis of the reserve components of the leaf blade, a rapid accumulation of soluble compounds occurs resulting in their translocation out of the blade into the petiole, and eventually on into the stem. The foregoing events undoubtedly accelerate respiration in the petiole, production of ethylene, a loss in the auxin gradient (**21**) across the petiole, digestion of the cell and cell wall materials, and cell division in the so-called abscission layer.

Finally, the results of the present paper do not support entirely the conclusions of V. L. Hall (**9**) that the starch or nitrogen content *per se* governs the susceptibility of the cotton plant to chemical defoliation. Rather it appears that the events that take place during the abscission process which

involve the rate of depletion of leaf carbohydrates and nitrogen, the accelerated respiratory rate, and the relative ratio between auxin and ethylene are as important, or more so, in regulating the course of abscission. In all fairness to V. L. HALL (**9**) it should be pointed out that he suggested that carbohydrate depletion is an important factor in the effectiveness of chemical defoliation and that there are other factors which determine the susceptibility of the cotton plant to chemical defoliation. It appears logical that the significance of a low starch content in defoliation is that it indicates the labile carbohydrates are exhausted and the reserve structural carbohydrates of the leaf are more subject to serve as enzymatic substrates under the influence of chemical defoliants. The writers' findings on the absence of a direct relation between carbohydrates and nitrogen and defoliation are in closer agreement with the final conclusions of EATON and ERGLE (**5**) although their work involved shedding of bolls in cotton. At the present time, there is no reason to believe that shedding and defoliation are basically different.

Summary

Results of experiments conducted in 1950 and 1951 which involve studies of compositional and physiological changes associated with the chemical defoliation of cotton are reported. Five techniques were employed in 1950 to alter the carbohydrate content of cotton leaves and study the effect upon the percentage defoliation obtained. In 1951 experiments were performed at four locations, and the mode of action of a uniform defoliant applied to plants grown under widely different cultural and environmental conditions was compared with plants defoliated with 100 p.p.m. of ethylene. Chemical changes in the carbohydrate and nitrogen fractions were followed by analyses at the time of defoliant application and thereafter at 24 hour intervals up to 72 hours during defoliation. Warburg respirometer measurements were made during abscission.

Little evidence was found for any correlation between leaf blade carbohydrates and the percentage defoliation effected in the 1950 work. There appeared to be more of an interaction between defoliant treatment and leaf composition during the defoliation process.

A similarity in the pattern of degreening (chlorophyll loss) of the leaf during natural abscission, defoliant-induced abscission and ethylene-induced abscission is illustrated and discussed.

No definite trends or correlations were found between the carbohydrate and nitrogen fractions of the leaf blade and the percentage defoliation obtained in 1951.

A rapid hydrolysis of the reserve constituents of the leaf blade and the polar transport of their soluble products occurred under the influence of the defoliant. The results with ethylene, although more pronounced, were comparable with those obtained with the defoliant.

The respiratory rate of the leaves sprayed with defoliant, when measured either by oxygen uptake or carbon dioxide production, approximately

doubled that of unsprayed leaves. The respiratory quotient indicated that carbohydrates were the primary substrates being oxidized under aerobic conditions.

The rapid hydrolysis and the rate of depletion of reserve compounds in the leaf blade and petiole brought about by the polar translocation of the mobile compounds as well as by oxidation in respiration, are believed to play a prominent role in chemically induced defoliation.

Based on the evidence of the present and other studies, a physiological course of events during chemical induced defoliation is proposed and discussed.

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SOME RELATIONSHIPS BETWEEN XEROPHYTISM AND THE CONTENT OF RESIN, NORDIHYDROGUAIARETIC ACID AND PROTEIN OF *LARREA DIVARICATA* CAV.

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(WITH ONE FIGURE)

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Introduction

Larrea divaricata is the dominant plant growing over more than 30,000,000 acres of the hottest and driest intermountain plains of the Mexican and southwestern American deserts. It is a remarkable plant, for although it is considered to be the typical xerophyte of all the deserts of these regions, it appears to be more similar to the mesophytes in morphology. RUNYON (**11**) has described the properties which adapt Larrea leaves to drought conditions as being added to an organization which to a considerable degree is mesomorphic.

Various hypotheses have been advanced to explain the drought resistance of the creosote bush but none appear adequate. Many investigators have credited the cuticular resins with the function of reducing transpiration (**6, 14, 16, 19, 20**) but ASHBY (**2**), SPALDING (**15**) and SCHRATZ (**13**) found the rate of transpiration unexpectedly high even in bushes having leaves sticky with resin. Ashby found no evidence of clogging of the very numerous and mesomorphic stomata. The fairly extensive root system of the creosote bush was considered partly responsible for its resistance to drought by some early workers (**5, 20**). This explanation is doubtful since the root system is rather similar to those of other species of angiosperms grown under semiarid conditions by DITTMER (**7**). MALLERY (**10**) has reported that the osmotic value of the cell sap of the leaves and small twigs of *Larrea divaricata* was especially high under drought conditions. However, BOON-LONG (**3**) has shown that even very high osmotic pressures do not reduce evaporation greatly.

RUNYON (**11, 12**) has observed that under conditions of extreme drought, the more mature leaves die and the immature leaves and buds dry out and turn brown. When more favorable moisture conditions exist again, the immature leaves and buds continue growth. According to Runyon, the creosote bush is the only seed plant known whose leaves have the ability of ceasing development before maturity, surviving intense periods of drought, and resuming growth. During the periods of extreme desiccation, transpiration continues at a low rate and diurnal variations of moisture content occur. Runyon could not find any significant morphological difference between the mature and the immature leaves. He reported the leaves of the creosote bush to be structurally similar to the mesomorphic plants, and

he suggested that the resistance to extreme drought might be related to physiological or chemical factors.

In the investigations described in this paper, the variations of certain chemical constituents of the creosote bush leaf were examined in an effort to determine their role, if any, in the drought resistance of the plant. Three constituents of the creosote bush appear to be of especial interest. One constituent is a phenolic compound, nordihydroguaiaretic acid (NDGA) which is concentrated in the cuticular resins and has never been reported in any other plant. A second constituent is the resin material which is present in an unusually high concentration (17% of ether extract). The third constituent is the hydrophilic protein. In this preliminary study the protein content was determined, and it is assumed that it should vary with the hydrophilic protein content.

There is evidence to indicate that chemical constituents of some desert plants do vary as the plants are subjected to increasing water stress. SPOEHR (**17**) found that in certain cacti, monosaccharide sugars decreased and pentosans and hexosans increased during summer drought. The pentosans imbibe water and apparently retard its loss. TRAUB *et al.* (**18**) reported a similar situation for guayule. They also found that during periods of exceptionally high moisture supply the plant apparently loses its ability to produce the polysaccharide inulin.

Experimental methods

The condition of the plant was classified as excellent when the foliage was lush and the leaves were deep green and succulent and poor when the foliage was sparse and the leaves were yellowish or brownish green and more or less brittle. The intermediate condition with green leaves was termed average. A plant in an individual location can be transformed from poor to excellent condition within a few days following a good rain. Leaf moisture varies directly with plant condition. Analyses show leaf and twig moisture to constitute 30, 40, and 73.5% of the air dry weight in plants in poor, average, and excellent condition, respectively.

The leaves and small twigs were collected from 12 individual plants including four each in excellent, average, and poor condition. The plants were collected within a radius of a few miles and were growing in soils of similar geological origin and parent material. Due to the arid history of the region, the soils had been subjected to little chemical weathering or leaching. The surface soils were light in texture (sandy loams), low in soluble salts (.02 to .07%) and slightly alkaline (pH 7.7 to 8.2). The principal difference found in the soils was the $CaCO_3$ content which varied from 0.6 to 12.1%. It is unlikely that the $CaCO_3$ would have any important effect on the plant constituents to be studied. MALLERY (**10**) could not find differences in calcium content in plants grown over caliche layers (cemented calcium carbonate) and those grown in less calcareous soils. The soil solution probably remained saturated in both cases. Analysis did not

indicate any relationship between the amounts of NDGA, resin and protein found in plants in the same condition and slight soil variations.

The leaves and small stems were separated from each plant, air-dried, ground and mixed. The method of DUISBERG *et al.* (**8**) was used for determination of NDGA. Resins soluble in alcohol were determined as the loss in sample weight after an eight-hour extraction with 95% alcohol. Preliminary studies showed that the extraction could be completed in four hours. The longer time was used to insure complete extraction in every case. Protein was determined by the Kjeldahl *et al.* method of the ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS (**1**).

Results

RESIN, NDGA AND PROTEIN IN INDIVIDUAL LEAF AND STEM SAMPLES

The results presented in table I are averages of the four samples with the standard errors. They show that alcohol-soluble-resin, NDGA, and

TABLE I

CHEMICAL ANALYSES OF CREOSOTE BUSH LEAVES AND STEMS IN PER CENT. DRY WEIGHT.

Plant part	Condition	Protein	Resin	NDGA
Leaves	Poor	14.16 ± 0.55†	35.91 ± 1.13	7.76 ± 0.33
	Average	13.83 ± 0.47	34.12 ± 0.78	7.46 ± 0.08
	Excellent	12.70 ± 0.70	36.80 ± 1.62	9.76 ± 0.36*
Stems	Poor	12.60 ± 0.46	26.44 ± 1.60	4.69 ± 0.67
	Average	11.99 ± 0.12	26.04 ± 1.11	5.20 ± 0.68
	Excellent	10.84 ± 0.76	28.56 ± 1.38	7.61 ± 0.16**

*Significant difference from other conditions at 1% level.
**Significant difference from other conditions at 5% level.
†Standard error.

protein are present in considerable amount in both leaves and small stems and are undoubtedly produced in both tissues. This observation confirms a finding of BOTKIN and DUISBERG (**4**) with respect to NDGA. In every case, the leaves contained larger amounts of each constituent than the corresponding stems. These differences were statistically significant at the 1% level. In both leaves and stems, the changes in chemical constituents as the condition of the plant improved were in the same direction and of the same relative magnitude. Plants in excellent condition were significantly higher in content of NDGA than those in the poorer conditions. The differences between the resin and protein contents of plants in different conditions were not statistically significant.

Correlation coefficients were calculated between the three constituents. The correlation coefficients between resins and protein were very low for leaves and stems. The correlation coefficient between protein and NDGA was negative and, although fairly high, was not statistically significant for

leaves and stems. The correlation coefficient between resins and NDGA was positive and almost high enough to be significant for both leaves and stems. In every case, the correlation coefficients for leaves and stems were of the same sign and of similar magnitude which would tend to increase the possible significance of the results. It is likely that a positive correlation exists between the resin and NDGA contents, since the NDGA is actually included in the resin fraction. More data are needed before conclusions can be drawn concerning the possible negative correlation between NDGA and protein.

Confirmatory evidence was obtained by analysis of 102 different samples collected at random in New Mexico, Texas, Arizona and Nevada over a five year period and during various seasons.

Contents of NDGA, resin and protein in regrowth samples

Three plants from which samples representing poor, average, and excellent conditions had been obtained were used to study regrowth. Regrowth

TABLE II

COMPARISON OF PROTEIN, RESIN AND NDGA CONTENTS OF REGROWTH WITH MATURE PLANTS IN PER CENT. DRY WEIGHT.

No. of samples	Condition	Protein	Resin	NDGA
4	Poor	13.21 ± 0.51	30.6 ± 1.80	6.05 ± 0.53
4	Average	12.91 ± 0.25	30.2 ± 0.62	6.40 ± 0.37
4	Excellent	11.89 ± 0.77	33.8 ± 1.05	8.90 ± 0.69
3	Regrowth	16.87 ± 1.32*	27.88 ± 0.54**	5.16 ± 0.27*

*Significant difference from mature plants at 1% level.
**Significant difference from mature plants at 5% level.

samples were collected two months after the plants had been cut to the bases. General summer rains giving three inches of precipitation began shortly after the plants were cut and there was vigorous regrowth of excellent condition in each case.

The samples of leaves and green stems of the regrowth averaged about 30 gm. in dry weight and were analyzed for NDGA, resin and protein. The results were similar for each constituent in all three regrowth samples, regardless of the original condition of the plant. The values for regrowth are compared with the calculated values for the leaves and stems of the other individual samples in table II. The protein content of the regrowth was higher and the resin and NDGA contents considerably lower than the values for mature plants in any of the three conditions. The differences are greatest when the regrowth is compared with plants in excellent condition, yet the regrowth was also in excellent condition. The only real difference between the regrowth samples and the mature plants in excellent condition lies in the maturity of the leaves. The statistical difference between the protein and NDGA contents of regrowth samples and the average of all of the

mature plants is well above the 1% level of significance. The statistical difference in the resin content almost reaches the 1% level.

Confirmatory evidence was obtained on a number of random samples which were collected in the spring after a period of new growth. Since many older leaves had dropped, these samples were fairly rich in new growth, but since they were not exclusively new growth, they can hardly be expected to yield differences as pronounced as those presented in table II. In figure 1, the random samples of new growth are compared with random samples of mature plants in excellent condition. The samples in excellent condition were used as a basis of comparison because the new growth would normally

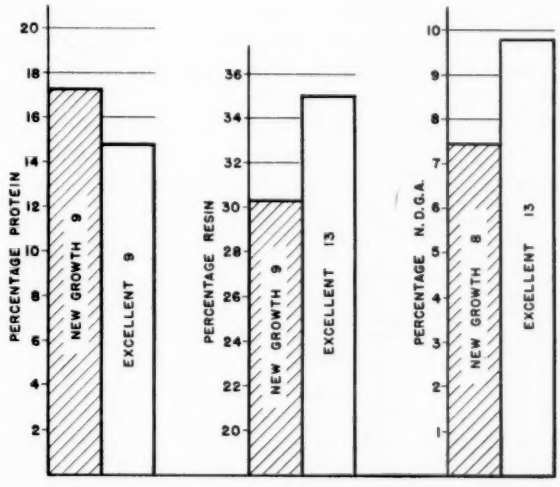

FIG. 1. Comparison of chemical constituents in new growth and plants in excellent condition on dry weight basis. Numbers in each bar refer to numbers of samples averaged.

be considered in excellent condition. If the average of all the random samples had been used, the differences would have been slightly less.

CHANGES IN NDGA, RESIN AND PROTEIN CONTENTS OVER AN EXTENDED PERIOD OF GROWTH

A number of samples were collected from nine different locations in the New Mexico Rio Grande Valley over a five year period. Each sample was hand-stripped and included leaves and twigs from about 30 representative plants. The data on the NDGA content of these samples have been reported previously (**4**).

The data collected over a four year period at the Fort Craig, New Mexico sampling area are of especial interest because the condition of the plants deteriorated progressively from good to very poor during three years of

drought. By the end of the third year the leaves were yellow and brown and the twigs brittle. Many plants seemed dead. Early spring rains in the fourth year resulted in a remarkable revival, and samples collected in May of that year were green and consisted almost entirely of new growth. The analytical results obtained over the four year period are presented in table III. Since the samples were collected by random sampling of a number of plants and leaves and stems were not separated, some variation may be expected. In spite of sampling limitations, the results show definite trends. The protein content remained relatively stable during the whole period of decline. In the new growth it attained a value about 7% above the highest previous figure. After the new leaves had matured, the protein value dropped toward normal. The resin content also remained fairly stable during the period of decline. In the new growth, it was lower than the lowest previous figure. After maturation of the new growth, the resin content reached a value slightly higher than normal. The NDGA content dropped appreciably from a rather high initial value as the plants changed from

TABLE III

ANALYSES OF CREOSOTE BUSH SAMPLES COLLECTED OVER A FOUR-YEAR PERIOD IN PER CENT. DRY WEIGHT.

Date collected	Condition	Protein	Resin	NDGA
July 17, 1945	Good	11.5	34.6	10.5
January 4, 1946	Fair to poor	11.8	34.6	9.0
April 19, 1946	Fair to poor	13.3	32.2	8.9
September 14, 1946	Poor	12.2	33.0	8.5
March 8, 1947	Very poor	13.2	33.7	8.4
October 18, 1947	Very poor	11.7	34.0	8.3
February 7, 1948	Almost dead	13.4	33.4	8.4
May 3, 1948	New growth	20.1	29.2	6.7
September 25, 1948	Fair	13.1	35.2	9.5

good to fair to poor condition. There was little further change as the plants deteriorated in condition over the next two years. The NDGA content of the new growth, however, was much lower than the lowest previous value. After the new growth matured, the NDGA content rose sharply to a rather high value.

Discussion

RUNYON (**11, 12**) classified the leaves of the creosote bush into two main groups on the basis of drought resistance. The leaves of the first group mature before the onset of drought conditions and generally do not have the ability to survive after the drought. The leaves of the second group which have a drought resistance unique among seed plant leaves, are immature and have been arrested in their development by drought conditions. These leaves have the extraordinary ability to resume growth on the advent of more favorable moisture conditions. In spite of their tremendous difference in physiological capacity, Runyon found the two leaf types to be similar in appearance, structure, and moisture content.

The present studies were of a preliminary nature and no attempt was made to separate the two types of leaves. Nevertheless, the regrowth samples were largely composed of young and immature leaves. The fact that the chemical differences between regrowth leaf samples and the mature-leaf samples were statistically significant for each of the three constituents studied indicates that the chemical differences between Runyon's leaf types may be considerably greater.

The higher protein content of the immature leaves may be related to the greater drought resistance of such leaves. It is reasonable to assume that the concentration of hydrophilic protein colloids in the cells would vary with the total protein content. Thus there would be a higher concentration of hydrophilic colloids in the cells of immature leaves with a consequent retardation in the rate of moisture loss during the later stages of desiccation. ILGIN (**9**) has shown that many types of plant cells can survive extreme desiccation if the drying process is gradual. At best, the protein data could furnish only a partial explanation for the drought resistance of the creosote bush because the leaves of many plant species that are not drought resistant have higher concentrations of protein.

The fact that NDGA and resin are formed in greatest amount in mature leaves which have been adequately supplied with water indicates that these substances are not related to the increased drought resistance of immature leaves, and that they are not formed in the plant as a response to drought conditions. The data from Fort Craig indicate that no significant changes occur in the concentrations of either constituent during prolonged periods of drought. The small decreases observed can be accounted for by the fact that the relative amount of stem tissue increased as the plant condition became poorer. It would appear desirable to investigate the precursors of resin and NDGA in immature leaves since the concentrations of both are low in new growth and increase as the leaves mature. These precursors may have a possible influence on drought resistance.

Summary

Samples of creosote bush foliage in conditions reflecting high, average, and low available moisture and regrowth samples were collected. The nordihydroguaiaretic acid, alcohol extractable resin, and protein contents of the samples were determined.

It was found that all three constituents are produced in considerable amount in both leaves and green stems. The leaves, however, were statistically higher in each constituent. The nordihydroguaiaretic acid contents of plants having access to abundant moisture were statistically higher than plants having access to less moisture. Resin also seemed highest in these samples. Since nordihydroguaiaretic acid and resins seem to be highest in plants having access to the most moisture there is no reason to believe that either constituent is formed in the plant as a response to drought conditions.

Immature leaves of the creosote bush differ from mature leaves and from all seed plant leaves in their remarkable ability to survive at very low

moisture contents and to resume growth after intense drought. Although there is no difference in morphology or moisture content, the immature leaves are lower in nordihydroguaiaretic acid and resin and higher in protein content. These chemical data suggest that basic chemical differences exist between the two leaf types.

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PHYSIOLOGICAL CHANGES AND FREEZING INJURY IN MATURING MAIZE[1]

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(WITH ONE FIGURE)

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The injurious effect of low temperatures on the viability of living organisms has been studied by many workers, and comprehensive reviews on the subject have been written by Levitt (**10**), Luyet and Gehenio (**13**), and Maximov (**14**). Extensive studies of freezing injury in maize have been published by Kiesselbach and Ratcliff (**9**) and Rossman (**18**). These workers found that moisture percentage of the grain was a major factor in injury along with temperature and duration of exposure. Since the moisture percentage of grains decreases with maturity, studies of freezing injury in these crops should be more valuable if accompanied by studies of physiological changes during maturity.

Earle *et al.* (**5**), analyzing the whole kernels of mature corn, found that the dry matter contained 1.7% sugars and 73.7% starch. In maturing corn, Culpepper *et al.* (**4**) observed that in both sweet and field corn kernels the reducing sugars decreased gradually. The sucrose was low at the beginning, then increased rapidly until 15 days after silking, and after that decreased slowly. Polysaccharides increased with age, but the rate of increase varied in different varieties. Evans showed (**6**) that the total amount of nitrogen in the corn kernel increased progressively until maturity was reached. On the dry matter basis, however, the percentage of total nitrogen decreased.

Maturity of maize is variously defined. The grain is generally considered ready to harvest at 20% moisture and safe from frost injury at 25%. Shaw and Loomis (**21**) have defined physiological maturity as the point at which maximum dry weight of grain is attained. Physiological maturity varies with variety and season between 25 to 30 and 40% moisture (**17, 18, 20**).

This work was planned to measure the effect of freezing on subsequent chemical and physiological changes in ears of maize treated to simulate freezing and drying in the field, with varying degrees of injury and at different stages of maturity.

Materials and methods

Single cross WF9 × 38-11 maize was used. Seed was planted on May 20 and May 23 in 1947 and 1948, respectively. Three stalks were left in hills 40 inches apart. In each year, the field was one plot, 8 rows by 80 hills. The whole plot was divided arbitrarily into two sampling areas or sub-plots.

[1] Journal paper no. J-2012 of the Iowa Agricultural Experiment Station. Project 1139.

The moisture content of the grain in the field was followed carefully and samples were collected at approximately 70, 60, 50, 40 and 30% moisture. As shown below, the fourth harvest at 40% moisture was physiologically mature in the 1948 experiments. Ears from each area were snapped at random with husks and shanks attached and divided randomly into four groups. One group of 10 ears was used immediately for moisture determinations of grain, husks, and cobs. A combined sample of kernels from the 10 ears was made; two samples each weighing 20 gm. were used for moisture determinations, and two samples each weighing 40 gm. were killed in 80% alcohol (**12**) for chemical determinations.

A second group of from 30 to 55 ears to be used as a control was spread on wire racks built between corn rows in the field. A third group of the same number of ears was put in the cold room immediately after harvest, exposed to a moderate freezing treatment, and then spread on the same racks. The object was to obtain injury or partial killing of the kernels. A fourth group of the same number of ears was given a more severe freezing to obtain almost complete killing. The resistance of ear corn to freezing increases rapidly as the grain matures and its moisture content decreases (**9, 18**). It was necessary, therefore, to increase the exposure and/or lower the freezing temperatures as the season progressed to obtain comparable physiological responses. Six to 10 ears from each group on the racks were taken to the laboratory for sampling and study after varying periods of drying and enzymatic change.

In 1947 the first harvest was made on September 1. A temperature of 20° F for eight hours was used for moderate freezing and 18° F for 16 hours for the more severe treatment. The second harvest was made September 7, the third on September 13, with treatments the same. The fourth harvest, on September 22, received more severe treatment, the moderate freezing being eight hours at 10° F and the severe one, 16 hours at 10° F. A fifth harvest of 10 ears was made on October 7 for final chemical samples, moisture, and kernel weight determinations.

In 1948, the general procedure in sampling was the same as in 1947, except that each time a combined sample of 10 ears was used for chemical samples, moisture, and kernel weight determinations. No determinations were made of the weights or moistures of husks and cobs.

The first harvest in 1948 was made on August 26. The moderate freezing was eight hours at 20° F, and the severe was eight hours at 0° F. The second harvest was made September 3 and received the same treatments. The third harvest was made on September 11. Treatments were eight hours at 20° F and 14 hours at 0° F for the moderate and severe freezings. The fourth harvest was made on September 19 with exposures of 20 hours at 20° F and 30 hours at 0° F. A fifth lot of 10 ears was collected October 5, but no freezing treatments were given.

The samples killed in boiling 80% alcohol were decanted four times, then extracted for 48 hours in Soxhlet extractors. For decantations and extractions, 80% alcohol was used for the 1947 samples and 70% for the

1948 samples. The grain was ground with a mortar and pestle before Soxhlet extraction. The extract of each sample was made to one liter volume and analyzed. The residues were dried at 100° C and ground to 60 mesh.

Reducing sugars, sucrose, and alcohol-soluble nitrogen were determined in the alcohol extract. For sugar analyses the extract was cleared by the method described by LIND (**11**). The procedure for reducing sugar and sucrose determinations in the cleared extract was that described by HASSID (**7, 8**). The method for nitrogen determination was a standard semimicro Kjeldahl using copper selenite as a catalyst. Germination tests in part of the work were made by Mrs. Van Kleek.

Results

The moisture percentages in both cobs and husks were higher than in the kernels at the early harvests. Moisture then declined rapidly in husks, and very slowly in the cobs in the later harvests compared to the kernels (table I). The dry matter per 100 kernels increased with maturity. In the

TABLE I

THE GREEN WEIGHT AND MOISTURE PERCENTAGE OF HUSKS AND COBS OF CORN AT DIFFERENT STAGES OF MATURITY, 1947.

Moisture in kernels	Husks		Cobs	
	Green weight	Moisture	Green weight	Moisture
%	*gm.*	%	*gm.*	%
64.9	137.0 ± 10.17	76.3 ± 0.98	193.1 ± 8.55	73.3 ± 1.09
58.3	88.8 ± 5.17	68.7 ± 0.74	139.3 ± 4.28	62.2 ± 1.39
50.3	37.4 ± 3.90	61.0 ± 2.31	108.5 ± 5.89	67.9 ± 1.03
39.5	22.2 ± 2.50	25.4 ± 3.55	110.3 ± 8.51	60.6 ± 1.52
31.8	25.0 ± 2.55	18.7 ± 1.92	110.8 ± 4.74	60.6 ± 0.70

1947 harvest, the dry matter per 100 kernels increased from 25 to 28.5 gm. when the moisture percentage in the kernels dropped from 40 to 30%. In the 1948 harvest of the same hybrid, however, no such increase was observed. ROBINSON (**17**) reported that maximum weight of 100 kernels of corn was attained when the moisture content dropped to 40%. On the other hand, SHAW (**20**) found that early lines of corn gained dry weight until the moisture dropped below 30%, but late lines were at full weight at 40%. It seems that the moisture percentage at attainment of maximum dry weight of the kernels depends both on factors within the plant and in the environment.

The kernels of frozen ears dried at a slower rate than the unfrozen when both were spread unshucked on wire racks between rows of corn in the field. An analysis of variance for 1948 data showed that the effect of freezing in slowing the rate of drying was highly significant. Also, the moderately frozen ears showed a significantly slower rate of drying than the severely frozen. Typical graphs are shown in figure 1.

The germinability of seed corn was greatly reduced by freezing. Previous investigators (**9, 18**) showed that the damage to seed corn by freezing

is increased with lower temperatures, longer duration, or higher moisture content of kernels at freezing time. In general, the same results were found here. When the green weights of seedlings produced by the corn were compared, freezing treatments reduced the average epicotyl weight, and severe freezing gave the lightest weight for epicotyls, considering each stage of harvest alone. Data for 1948 are presented in table II. It should be noticed that temperatures and durations were not the same for the different stages of maturity.

In the 1947 crop, the percentage of reducing sugar in immature corn was 1.61 at 65% moisture, then dropped to 1.15, 0.87, 0.62, and 0.48 for the second, third, fourth, and fifth harvests, respectively. The percentage of sucrose was much higher at each stage. For the first harvest, the percent-

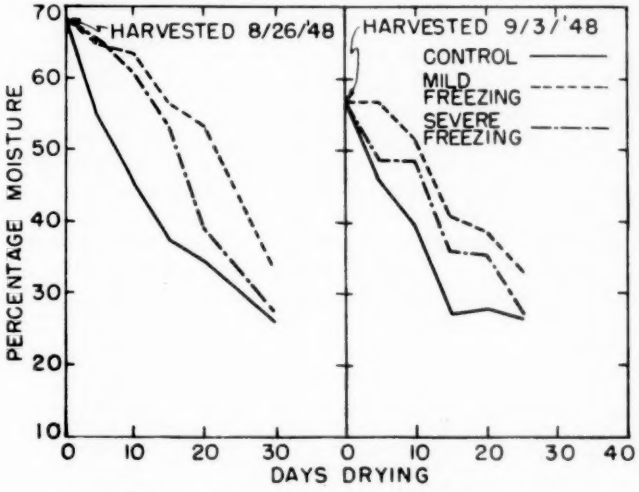

FIG. 1. Rate of drying of ears of corn as affected by freezing.

age of sucrose was 3.98 and it decreased gradually to 1.32 for the fifth harvest. In the 1948 crop the same trend occurred.

When the ears were dried in the field the percentages of both reducing sugars and sucrose decreased gradually. The rate of decrease of reducing sugars, however, was significantly slowed down by freezing. The moderate and severe freezing had about the same effect on the rate of decrease of reducing sugars. On the other hand, freezing accelerated the rate of decrease of sucrose significantly, and severe freezing had more effect than moderate. Data for 1948 are presented in table III.

BLISH (3) and NEWTON and MCCALLA (16) found that frozen wheat contained more reducing sugars than unfrozen after drying, but sucrose was not affected. KIESSELBACH and RATCLIFF (9) found no difference in the chemical constituents of frozen and unfrozen corn grain. Previous work on

TABLE II

EFFECT OF FREEZING ON GERMINATION AND ON GREEN WEIGHT OF EPICOTYLS, 1948.

Moisture at harvest	Germination			Average fresh weight of 100 epicotyls		
	Control	Moderate freezing	Severe freezing	Control	Moderate freezing	Severe freezing
%	%	%	%	gm.	gm.	gm.
68.5	99	32	5.5	60.4	46.5	37.3
56.9	98	76	41.0	66.0	59.1	54.9
46.6	98	81	2.5	78.4	64.8	44.0
37.8	100	75	7.0	88.4	65.6	60.3

corn by the writer (**1**), however, showed results similar to those reported here.

Data for the 1947 and 1948 crops showed that the percentage of total nitrogen in the dry matter of maturing kernels decreased steadily and slowly until development was completed. This result is in agreement with those of EVANS (**6**). The percentage of alcohol-insoluble nitrogen remained constant while that of the alcohol-soluble decreased gradually. ZELENY (**22**) made a detailed study on various nitrogen fractions in the developing corn kernels. His data indicate that water-soluble, non-protein nitrogen, as a percentage of total nitrogen, decreased with maturity. The same was true with proteoses, but the prolamine nitrogen (zein) increased and the glutelin nitrogen showed a slight increase, while the globulin remained nearly stable. The percentage of alcohol-soluble nitrogen decreased when the ears were dried in the field after harvest, while that of insoluble and total nitrogen in-

TABLE III

THE CHANGES IN SUGARS IN PER CENT. DRY WEIGHT IN MATURING CORN KERNELS AS AFFECTED BY DRYING AND FREEZING, 1948.

Moisture at harvest	Days drying	Reducing sugar			Sucrose		
		Control	Moderate freezing	Severe freezing	Control	Moderate freezing	Severe freezing
%		%	%	%	%	%	%
68.5	0	2.63	2.63	2.63	5.33	5.33	5.33
	5	1.00	1.52	1.74	1.24	1.03	0.84
	10	0.76	1.28	1.09	1.34	1.02	0.57
56.9	0	1.19	1.19	1.19	3.23	3.23	3.23
	5	0.87	0.87	0.84	1.08	0.73	1.51
	10	0.61	0.65	0.58	1.01	0.85	0.50
46.6	0	0.86	0.86	0.86	2.83	2.83	2.83
	5	0.64	0.69	0.87	1.08	0.78	0.30
	10	0.43	0.50	0.58	0.98	0.95	0.28
37.8	0	0.70	0.70	0.70	2.13	2.13	2.13
	5	0.48	0.87	0.71	1.01	1.50	0.60
	10	0.62	0.78	0.70	1.03	1.11	0.59
24.8	0	0.50	0.50	0.50	1.24	1.24	1.24

creased. Analysis of variance for the 1948 data indicated that the increase in total and insoluble nitrogen with drying and the decrease in the alcohol-soluble nitrogen were all highly significant. A comparison of the effect of the second five days of drying indicated that there was a change in both alcohol-soluble and insoluble nitrogen during this period and there was no change in the total nitrogen. Most of the change in total nitrogen occurred in the first five days.

Freezing slowed both the decrease of alcohol-soluble nitrogen and the increase of insoluble and total nitrogen with drying. In a comparison between the effect of moderate and severe freezing on the nitrogen fractions, a significant difference was found for all fractions except total nitrogen. Data for 1948 are shown in table IV.

TABLE IV

CHANGES IN NITROGEN FRACTIONS IN PER CENT. DRY WEIGHT OF MATURING CORN KERNELS AS AFFECTED BY DRYING AND FREEZING, 1948.

Moisture at harvest	Days drying	Nitrogen								
		Alcohol soluble			Insoluble			Total		
		Control	Moderate freezing	Severe freezing	Control	Moderate freezing	Severe freezing	Control	Moderate freezing	Severe freezing
%		%	%	%	%	%	%	%	%	%
68.5	0	0.931	0.931	0.931	1.453	1.453	1.453	2.384	2.384	2.384
	5	0.934	0.826	0.964	1.804	1.590	1.422	2.738	2.416	2.386
	10	0.733	0.836	0.943	1.769	1.640	1.575	2.502	2.476	2.518
56.9	0	0.713	0.713	0.713	1.452	1.452	1.452	2.165	2.165	2.165
	5	0.491	0.706	0.656	1.634	1.535	1.474	2.125	2.241	2.130
	10	0.457	0.392	0.551	1.942	1.835	1.689	2.399	2.227	2.240
46.6	0	0.483	0.483	0.483	1.412	1.412	1.412	1.895	1.895	1.895
	5	0.343	0.306	0.562	1.585	1.594	1.593	1.928	1.900	2.155
	10	0.380	0.416	0.514	1.424	1.439	1.477	1.804	1.855	1.991
37.8	0	0.329	0.329	0.329	1.450	1.450	1.450	1.779	1.779	1.779
	5	0.195	0.422	0.429	1.662	1.517	1.436	1.857	1.939	1.865
	10	0.239	0.380	0.481	1.659	1.674	1.456	1.898	2.054	1.937
24.8	0	0.187	....	....	1.393	....	....	1.580	....	....

Discussion

In previous work (1) the dry weight for 100 kernels was found to increase from 24.8 gm. at 40% moisture, to 28.3 gm. at 35.4% moisture, and to 32.3 gm. at 24.9% moisture in early plants. In late plants of the same variety, the weight was 22.2 gm. at 40% moisture and remained the same at 26.8% moisture. The dry weight of 100 kernels increased after the moisture dropped below 40% in the 1947 crop, but such an increase in dry weight was not observed in the 1948 crop. ROSSMAN (18), employing dry weight per kernel, showed that the dry weight of all varieties he used increased when the moisture was below 40%. From these results and others

obtained by SHAW (**20**) it may be stated that the maximum dry weight is not necessarily attained when moisture reaches 40% but is affected by varietal and seasonal differences.

When green ears are frozen, the husks are killed and dry quickly. They tend to stick to the ear and do not expose it to the atmosphere as do the unfrozen husks. The dried shucks tightly enveloping the ear decrease the rate of drying. The slower drying rate of frozen corn shown in figure 1 was not obtained with shucked ears.

Other workers have shown that corn kernels contain more sucrose than reducing sugars throughout their development, and that both sugars decrease with maturity. These results are confirmed here. It may be added also that sugars in harvested ears decreased greatly on drying. The rate of decrease in reducing sugars was fairly slow. Sucrose, however, decreased rapidly at the beginning, then more slowly.

Data in these experiments for 1947 and 1948, and previous work (**1**) show that the reducing sugars were higher in the frozen corn than in the controls but sucrose was lower. The changes in the sugars of frozen corn kernels during drying indicate that enzymes responsible for these changes were still functioning. Enzymes of various types are known to withstand low temperatures. BAILEY and GURJAR (**2**) suggested that the disorganization of the protoplasm of wheat by frost resulted in the activation of the hydrolytic enzymes and led to an accumulation of split products. MCCALLA and NEWTON (**15**) accepted this hypothesis on the basis of their results in wheat. The rapid loss of sucrose in corn kernels may have been a result of increased hydrolysis that took place in frozen kernels.

As indicated above, the frozen ears contained more alcohol-soluble and less insoluble nitrogen after drying than the unfrozen, the total nitrogen being the same in both. MCCALLA and NEWTON (**15**), working on wheat, believed that respiration was reduced in the frost-injured germ during subsequent drying, and thus a higher C/N ratio occurred in dried kernels. The same injury checked synthesis and resulted in larger fractions of non-protein and salt-soluble nitrogens. SHARP (**19**) believes that the synthetic reaction of complex proteins from amino compounds is normally irreversible, and that this reaction is rendered reversible by freezing, with a tendency to form more amino compounds. The picture of nitrogen distribution in frozen corn kernels may be drawn as follows. When the unfrozen ears are left to dry, nitrogen in simple forms is translocated from the cob into the kernels where it is synthesized into more complex forms such as zein, and particularly glutelins and globulins. If ears are frozen, the translocation does not cease but the rate of synthesis is decreased if not completely stopped.

The decrease in germinability of frozen seed corn has been shown to depend on many factors (**9, 18**). Data from these experiments show that the fresh weight of seedling epicotyls increased with maturity and, within any one harvest, decreased with the degree of freezing injury. It seems probable that reduced vigor may be due either to quantitative or to quali-

tative properties of the protoplasm and nutrients. The reduction in vigor of immature seed may be due to less protoplasm in the embryo and to fewer reserves; the reduction in vigor of frozen seed may be due to the change of protoplasmic properties of the embryo, and to a reduction of the food supply by the killing of sections of the endosperm.

Summary

The physical and chemical changes in dried kernels from ears harvested at different stages of development were compared with those occurring in frozen kernels. Maximum dry weight of kernels was attained at different moisture levels, depending on variety and season. Under field conditions, the ears snapped and frozen dried more slowly than the unfrozen. Both reducing sugars and sucrose decreased in the kernels with maturation. In the drying ears, sucrose in kernels decreased rapidly but reducing sugars decreased slowly. When ears were frozen before drying, the kernels contained less sucrose but more reducing sugars. During maturation, the alcohol-soluble nitrogen and the total nitrogen percentages decreased while the insoluble nitrogen remained constant. With field drying, the alcohol-soluble nitrogen decreased, while both the insoluble and total nitrogen increased. The frozen ears, however, contained more alcohol-soluble nitrogen, less insoluble, and the same percentage of total nitrogen as the unfrozen. Freezing slowed the synthesis of protein but apparently did not stop nitrogen translocation from cobs and shanks.

Ability of corn kernels to germinate starts early in their life, but the more mature seed produced more vigorous seedlings. Freezing decreased both germinability and viability of seed corn. Reduction of vigor may be due either to protoplasmic injury or to a reduction in quantity or availability of seed reserves.

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A RELATIONSHIP BETWEEN PHOTOPERIODISM AND RESPIRATION[1]

B. B. Elliott and A. C. Leopold

(with three figures)

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Introduction

The discovery by Garner and Allard (**3**) that many plants have specific photoperiodic requirements for floral initiation has led to considerable research to determine the effect of light in the transition from a vegetative to a flowering condition. It might be expected that this transition would be reflected in some fundamental process in metabolism, possibly in respiration. The results obtained by Melchers and Lang (**7**) indicate that some metabolic function, presumably a catabolism of sugar, is involved in the flowering response of *Hyoscyamus niger*, a long-day plant. While Bode (**1**) has reported changes in respiration and photosynthesis, and Roberts *et al.* (**9**) have found respiratory changes associated with photoperiodic induction, the results of these workers were obtained long after the flower perimordia had been initiated.

It is the purpose of this study to investigate the metabolic changes, as expressed by respiratory activity, which may be associated with the onset of floral initiation.

Materials and methods

The material in the present study included two short-day plants (cocklebur and Biloxi soybean), two indeterminate plants (Alaska pea and tomato var. Michigan State Forcing), and a long-day plant (Wintex barley). Unless otherwise stated, the experimental material was grown in the greenhouse under a day length that would maintain a vegetative type of growth until the plants were sufficiently mature for experimentation. The plants were then transferred to chambers with controlled temperature and light conditions, and subjected to the photoperiod treatments indicated in table I.

The treatments involving night interruption consisted of day lengths one hour shorter than the short-day treatment with one hour of light added in the middle of the dark period or night. These treatments were particularly useful in providing material comparable to the short-day treatment in hours of illumination and comparable to the long-day material in photoperiodic stimulus. It is well established that a light interruption in the middle of the night period negates the effect of a long dark period both in long-day plants (**2**) and in short-day plants (**5**).

Mature leaves were cut from the plants, brushed lightly to remove contaminants, and circular disks were cut from them. The leaf disks were

[1] Journal paper no. 547 of the Purdue University Agricultural Experiment Station.

TABLE I

THE DURATION OF THE LIGHT PERIODS GIVEN TO THE VARIOUS PLANTS.

Plant	Short day	Night interruption	Long day
	hrs.	*hrs.*	*hrs.*
Cocklebur	14	13 + 1	16.5
Biloxi soybean	10	9 + 1	14.0
Alaska pea	10	13 + 1	16.5
Tomato var. Michigan State Forcing	10	13 + 1	16.5
Wintex barley	10	9 + 1	12.0

placed in conventional Warburg flasks and their oxygen consumption was determined by the method of KLINKER (**6**). Temperature was held at 27° C.

Preliminary experiments indicated that maximal differences in respiration between treatments occurred at about midday. Thus all respiration readings were started at about 1 P.M. The duration of all respiration determinations was one hour. The leaf disks were then removed from the flasks, placed in a constant temperature oven at 75° C for 15 hours, and weighed. The results of the various experiments are expressed as the Q_{O_2}, *i.e.*, the microliters of oxygen taken up per milligram of dry weight of tissue per hour.

After each series of photoperiod treatments, a group of 7 to 10 plants was transferred to a non-inducing day length, and after three weeks the number of flower primordia was determined by micro-dissection.

Results

THE EFFECT OF PHOTOPERIODS ON THE RESPIRATION OF SHORT-DAY PLANTS

To establish the effect of the light period on the respiratory processes of some short-day plants, Biloxi soybean and cocklebur were subjected to short-day, long-day and night-interruption treatments. The results are shown in table II. It can be seen that in the case of cocklebur, one short day followed by a long night resulted in a decreased rate of respiration,

TABLE II

THE RELATIONSHIP OF PHOTOPERIODISM TO THE RESPIRATION OF SPORT-DAY PLANTS, COCKLEBUR AND BILOXI SOYBEAN. THE Q_{O_2} IS EXPRESSED AS PER CENT. OF THE LONG-DAY CONTROL (VEGETATIVE).

Plant	Days of treatment	Q_{O_2} short day	Q_{O_2} night interruption
Cocklebur	1	90	83
	2	111	97
	3	118	96
Biloxi soybean	1	111	90
	2	123	90
	3	143	90
	4	145	108
	6	119	93

while two or three such photoperiods produced increases up to 18% over the vegetative control. Biloxi soybean did not show an initial depression but exhibited an increase in respiration rate with each short day up to the third day, at which time a maximal increase was observed. Thus, there was an overall increase in respiration rate with floral induction both in cocklebur (18%) and in Biloxi soybean (45%) compared to that of vegetative controls. The night-interruption experiments failed to exhibit an increase, except at one point (Biloxi soybean treated four days), and this difference was too small to be significant. It has, thus, been demonstrated that the photoperiodic induction of flowering in these two short-day plants was associated with an increase in respiratory activity. The same number of

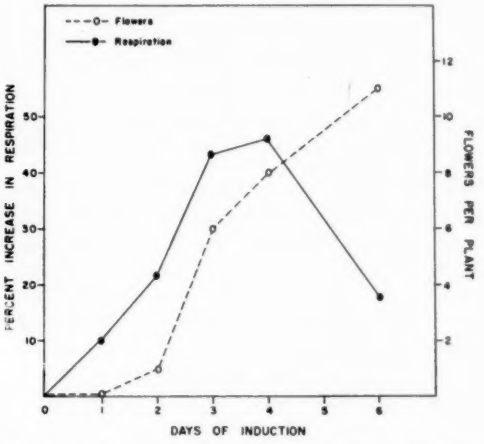

FIG. 1. The effect of number of days of induction on the respiration rate and on the number of flowers induced in a short-day plant, Biloxi soybean.

light hours given as a non-inducing, night-interruption treatment did not evoke such an increase in respiratory activity.

Counts of the number of flower primordia induced in soybean by each number of short days were made after three weeks. The relative effect of each number of short days on respiration rate and on flower induction are compared in figure 1. It can be seen that the greatest rise both in the respiration rate and in the number of flowers ultimately induced occurred with the third day of induction.

THE EFFECT OF PHOTOPERIODS ON THE RESPIRATION OF A LONG-DAY PLANT

To establish the nature of the respiratory response of a long-day plant to photoperiods, Wintex barley was subjected to long-day, short-day, and night-interruption treatments. Figure 2 indicates the respiratory responses

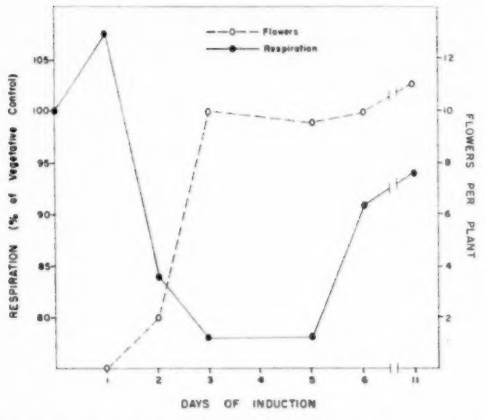

FIG. 2. The effect of number of days of induction on the respiration rate and on the number of flowers induced in a long-day plant, Wintex barley.

exhibited by Wintex barley subjected to long days. It will be observed that a single inducing day-length resulted in an increased respiratory activity whereas subsequent days of induction resulted in decreases up to 21% below the rate of the vegetative control. These results are in contrast to those shown with the short-day plant in figure 1. It appears that the maximal changes in both the flowering and respiratory activities occurred on the second and third days of induction.

In the case of the night-interruption treatments, which are presented graphically in figure 3, it will be observed that while there was a lag in the

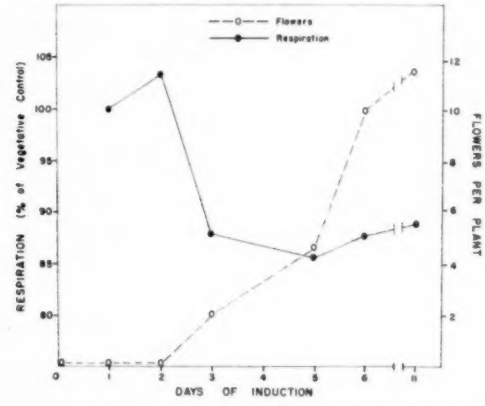

FIG. 3. The effect of number of interrupted nights on the respiration rate and on the number of flowers induced in a long-day plant, Wintex barley.

respiratory response, the number of flowers per plant closely paralleled the respiratory shift. It appears that the night interruption treatment was less effective both in inducing the formation of flowers and in decreasing respiration rate.

It may be stated that at least in the case of Wintex barley there is an overall decrease in the respiration rate with flower initiation. The decrease is observed whether flower initiation is brought about by long photoperiods or by short photoperiods with night interruptions. This decrease is in contrast to the increase in respiration rate demonstrated with the short-day plants tested.

The effect of photoperiod on the respiration of indeterminate plants

To determine if the respiratory shifts which had been observed with both the long-day and short-day plants were related to strictly developmental phenomena in the plants, two day-neutral or indeterminate plants, Alaska pea and tomato (var. Michigan State Forcing) were subjected to the three light treatments. The results shown in table III indicate consistently higher respiratory activities in the long-day treatment. Furthermore, the respiration rates of the plants receiving the night-interruption treatment are essentially identical with those of plants receiving the short-day treatment which is very much in contrast to the behavior of the photoperiod-sensitive species.

TABLE III

THE RELATIONSHIP OF PHOTOPERIODISM TO THE RESPIRATION OF INDETERMINATE PLANTS, ALASKA PEA AND TOMATO (VAR. MICHIGAN STATE FORCING).

Plant	Days of treatment	Q_{O_2} short day	Q_{O_2} night interruption	Q_{O_2} long day
Alaska pea	1	4.31	4.33	5.00
	3	4.63	4.55	6.50
	5	4.68	4.75	6.83
Average		4.54	4.54	6.11
Tomato	1	2.67	2.63	2.94
	3	2.72	2.68	3.15
	5	2.77	2.59	3.09
Average		2.72	2.63	3.06

Discussion

On the basis of the experimental evidence presented here, it is proposed that the photoperiodic mechanism by which flowering is induced involves a respiratory shift. There are three lines of evidence to support this hypothesis. First, it has been shown with one long-day and two short-day plants that there is a shift in respiration rate with photoperiodic induction; and

upon being induced, the short-day plants exhibited an increase in respiration rate, while the long-day plant exhibited a decrease in respiration rate when compared to their vegetative controls. Second, the night-interruption treatments evoked respiratory rates comparable to the rates in long days in all of the photoperiod-sensitive species tested but not in the indeterminate species, which parallels the effects of night interruption on flowering. And third, a close similarity was found to exist between the flowering and respiratory patterns associated with different numbers of photoinducing treatments.

Long-day and short-day plants exhibited opposite flowering responses to the light interruption of a long dark period. Night interruption of short-day plants did not induce flowering whereas night interruption of the long-day plant did induce flowering. Similarly, night interruption of the short-day species tested did not result in a respiratory shift, whereas night interruption of a long-day plant did evoke such a shift. Night-interruption treatment of indeterminate plants does not alter flowering, and no change in respiration rate was observed.

A relationship has been shown to exist between respiration and photoperiodism at the onset of floral initiation in the photoperiod-sensitive species tested. The present data provide fundamental evidence of the existence of metabolic processes which may be directly involved in the mechanism of photoperiodism in plants.

Summary

Changes in respiratory rates with various photoperiod treatments have been investigated using short-day plants (cocklebur and Biloxi soybean), indeterminate plants (Alaska pea and tomato var. Michigan State Forcing), and a long-day plant (Wintex barley). Respiratory activities expressed as the Q_{O_2} have been determined in long-day, in short-day and in short-day with night-interruption treatments. The results indicate that the respiration rates in leaf disks of long-day plants decreased and those of short-day plants increased with photoinduction. In contrast to these results, the respiratory activities of the indeterminate plants appear to be comparable to the total light received.

The data presented exhibit a correlation between the extent of changes in respiratory activities and the degree of flowering in response to the first few days of photoperiodic induction.

On the basis of the experiments reported, a hypothesis is offered that the photoperiodic mechanism by which flowering is induced involves a respiratory shift.

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SOME EFFECTS OF ARTIFICIAL DRYING OF CORN GRAIN[1]

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Artificial drying is commonly used for conditioning corn harvested for seed. In addition, artificial drying is being applied more frequently to market corn which has been (a) damaged by early frost; (b) harvested under weather conditions unfavorable for storage; (c) harvested before natural drying was complete, because of a common tendency to use mechanical harvesters early; or (d) harvested early to take advantage of high prices when grain is scarce. With the advent of artificial drying, inquiries have arisen as to its effect on the nutritional and commercial value of the dried grain. Smith *et al.* (**10**) concluded that the lowered industrial value of artificially dried corn may be due to lack of uniformity in drying. Bartling (**3**) found that artificial drying renders corn brittle. Cox *et al.* (**5**) reported difficulty in processing corn dried at 180 to 200° F because the protein adhered to the starch more tenaciously than in air-dried corn. McHargue (**9**) found some glyceride hydrolysis with drying temperatures of 75 and 98° C. The purpose of the present study was to determine the effect of various artificial drying conditions on the composition and ease of processing corn.

Methods

U.S. 13, an adapted double-cross corn hybrid, and its component single crosses, WF9 × 38-11 and Hy × L317, and inbreds, WF9, 38-11, Hy, and L317, were planted May 15, 1949, in a randomized block design with five replications. Sampling was begun approximately 20 days after pollination at which time the corn caryopses had a water content of 80%. Immediately after the ears were removed from the plants, initial moisture was determined by removing four kernels from two sides of the tip, shank end, and center of each ear. In high-moisture corn, the kernels were carefully removed from the cob with a knife.

The corn samples were dried as ear corn in an experimental drier under controlled conditions of humidity, temperature, and air flow. The temperatures used were 181.2 ± 1.5° F, 129.0 ± 2.5° F, and 109.8 ± 1.0° F which will be referred to as high, medium, and low, respectively. The chemical and processing data were obtained on U.S. 13 only.

The drying apparatus consisted of a closed duct through which air was circulated by a centrifugal blower. The part of the duct in which the corn sample was placed consisted of a vertical section with inside dimensions

[1] Published with the approval of the directors of the Illinois Agricultural Experiment Station and the Northern Regional Research Laboratory.

19 × 19 inches. A smaller duct of inside dimensions, 6 × 10 inches, was joined to the vertical section above and below the position of the sample to be dried, to complete the air circuit. The blower was installed in the small section of the duct. The corn sample was carried in a tray suspended from a Toledo scale so that weight of the sample could be determined at any time during the test. The ears were held in the tray in a vertical position, their axes being parallel to the air flow so that the entire surface was equally exposed. The sides of the tray were solid and the bottom was of small-mesh hardware cloth. There were places for 36 ears in the tray, but only sufficient ears were used to provide the amount of corn desired for each run.

In order to get accurate duct temperatures, a thermocouple probe was used to determine the temperature at the center of each of 12 equal areas of the duct cross section at a point before the air came in contact with the sample. Both dry bulb and wet bulb thermocouples were used so that relative humidities could be obtained. These humidities were approximately 10% at high temperatures, 20% at medium temperatures, and 30% at low temperatures. The wet bulb thermocouple was kept moist by water from a reservoir held above the thermocouple. Traverses to obtain accurate average wet and dry bulb temperatures were made at three-hour intervals. To determine the constancy of wet and dry bulb temperatures, fixed thermocouples in the traverse cross section were used to record the temperatures continuously. The instrument recording the dry bulb temperature also regulated the air temperature by controlling an electric heating element located in the small section of the duct.

The entire duct system was built of asbestos cement board one half inch in thickness and was not completely air-tight, allowing a small amount of air to pass from the duct. Some air, laden with moisture, escaped through the walls of the system and this was replaced by drier air from the room which tended to prevent the humidity of the circulating air from increasing. It was usually necessary to add moisture to the circulating air at intervals to compensate for the moisture lost through the duct walls. The addition of moisture was accomplished by generating steam with an electric heater immersed in distilled water in a pipe connected to the duct. The air flow was regulated by pulleys giving suitable blower speeds. The rate of flow was measured in the small section of the duct by means of a Thomas meter which measures the electric energy required to heat the air 2° F. The air flow did not vary appreciably in the tests, having a velocity of approximately 0.85 feet per second.

The ear corn placed in the drying chamber was dried until the moisture content of the kernels was reduced to approximately 13%. The corn was then shelled and spread on a screen for 48 hours before storage. Final moistures were determined at 100° C in a forced-draft oven. Controls were air-dried by hanging in bags in a ventilated room which had an average temperature of 75° F. Samples to be lyophilized were frozen in the field with dry ice immediately after removal from the plant.

Chemical analyses were by the methods of the ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS (**1**) with the exception of starch which was determined by CLENDENNING's polarimetric method (**4**). Vitamins, with the exception of carotene, were determined by microbiological procedures (**8**). All analytical data are reported on a moisture-free basis.

Investigations on germination and viability were made in a randomized block design with four replications each in the greenhouse and seed germinator. The triphenyltetrazolium chloride color test for germinability also was used (**2**).

Corn which had been artificially dried in 1949 was tested for field performance by planting (May 23, 1950) in a randomized block design with four replications. Plots were 2×5 hills in size. Controls were air-dried samples with the same intial moisture content as that of the corn artificially dried. Five kernels per hill were planted, and later each hill was thinned to two plants. Yield of grain and average height of plants were used as criteria of performance.

For the determination of starch viscosity, one liter of a 5% (dry basis) starch suspension was used for each run. The slurry was stirred vigorously and immediately poured into the beaker of a viscometer and run approximately 20 minutes, during which time pasting was completed. The pH of the starch slurry was determined before and after each run.

In the wet-milling process in the laboratory, 400 grams of corn were placed in a 2-liter Erlenmeyer flask, with the bottom drawn to form a cone, and steeped for six hours at 49° C in one liter of a circulating aqueous steep solution containing 0.05% sulphur dioxide. At the end of the six hours, a 0.2% sulphur-dioxide solution was slowly fed into the steeping flask at a rate of one liter per 18 hours. To maintain a constant volume in the steeping flask, an equal amount of steeping solution was removed from the flask by a siphon system. The dried, immature, corn samples absorbed most of the initial steeping solution. In such cases, 200 ml. of 0.2% sulphur-dioxide solution were added directly to the steeping flask at the end of six hours. At the end of seven hours, one liter of 0.2% sulphur-dioxide solution was gradually fed into the flask over a period of 17 hours. After the 24-hour steeping period, the steep solution was drained off and the corn ground three times through a laboratory mill. The starch and gluten fraction was then separated from the fiber fraction by sieving the ground slurry (200-mesh copper screen) four times. The combined slurries containing the starch and gluten were concentrated by centrifuging. The gluten was removed from the starch by first shaking the concentrated slurry, then centrifuging it, decanting the supernatant water, and scraping off the gluten layer which lay above the starch. More water was added to the partially cleaned starch and the centrifuging, decanting, and scraping cycle was repeated four times. The cleaned starch was removed from the centrifuge bottles and dried to approximately 12% moisture content in a forced-draft oven at 40° C.

TABLE I

REDUCTION IN GERMINATION, UNDER GERMINATOR CONDITIONS, OF INBREDS AND SINGLE CROSSES DRIED AT 110° F COMPARED WITH AIR-DRIED CONTROLS HAVING THE SAME INITIAL MOISTURE CONTENT.

Inbred or single cross	Initial moisture	Reduction in germination
	%	%
WF9	45.0	6
38-11	41.0	2
WF9 × 38-11	37.9	3
Hy	43.1	14
L317	44.8	93
Hy × L317	34.9	1

Results and discussion

High drying temperatures produced a marked reduction in the viability of U.S. 13. As found by DIMMOCK (**6**), working with single crosses and inbreds, a differential response was made by the component single crosses and inbreds of U.S. 13, all of which failed to germinate after a drying temperature of 130 or 180° F. It is especially significant that L317, dried at 110° F, showed lower germination than the air-dried control (table I).

Vigor likewise is influenced by maturity and drying temperatures. DUNGAN (**7**) found that mature corn germinated more slowly, but was more vigorous and had more secondary roots and longer shoots than immature corn. Drying of high-moisture corn promoted radical growth but delayed the plumule growth of U.S. 13 on the germinator. This effect was also apparent in the delayed emergence and stunted growth found in investigations conducted in the greenhouse.

Table II presents data on the field performance of corn dried at 110° F and corn dried at room temperature. Before drying, both had the same ini-

TABLE II

A COMPARISON OF THE FIELD PERFORMANCE OF CORN, AIR-DRIED AND ARTIFICIALLY DRIED AT 110° F, HAVING THE SAME INITIAL MOISTURE AT TIME OF HARVEST. DATA ARE GIVEN AS PER CENT. OF AIR-DRIED CONTROLS.

Variety	Initial moisture when harvested	Yield	Normal height of plants July 5	Normal height of plants September 14
	%	%	%	%
U.S. 13	39.0	84.5	90.5	97.2
U.S. 13	27.6	91.6	97.3	99.0
U.S. 13	46.6	90.4	55.3	98.1
WF9	45.0	81.9*	99.0	99.1
Hy	43.1	78.5*	91.2	97.2
38-11	41.0	121.0	96.6	100.0
WF9 × 38-11	37.9	84.7*	80.6	99.1
Hy × L317	34.9	92.5	93.0	98.2

*Significant at the 5% level.

TABLE III

COMPOSITION OF IMMATURE CORN GRAIN DRIED IN DIFFERENT WAYS.

Method of sampling	Initial moisture	Ash	Protein (N × 6.25)	Oil	Sugar	Starch
	%	%	%	%	%	%
Air-dried	68.5	2.19	12.8	2.7	2.11	65.0
Lyophilized	69.0	2.84	13.1	4.3	13.61	55.5
Lyophilized and oven-dried*	69.0	2.59	12.8	4.0	10.00	57.8

*Samples were lyophilized and oven-dried at 100° C for 48 hours.

tial moisture content. Artificial drying of inbreds WF9 and Hy, and the single cross, WF9 × 38-11, caused them to give significantly lower yields than the control; whereas the yield of inbred 38-11 was not reduced by similar treatment. Drying at 110° F caused a reduction in plant height of all strains treated, as determined by measurements made on July 5. Some lots of seed showed a greater reduction in height than others. U.S. 13, with 46.6% initial moisture, was severely affected by the drying. However, by September 14, when plant height was again taken, these differences had practically disappeared. There was no correlation between reduction in yield and reduction in plant height due to artificial drying at 110° F.

Artificial drying rendered corn brittle; corn harvested at high-moisture content and dried at 180° F was most brittle. Brittleness resulting from drying at a high temperature decreased as the corn matured. Below 50% moisture there was little change in brittleness caused by artificial drying. In the air-dried samples, maturity had no effect on brittleness.

The influence of three methods of drying upon the composition of immature corn is given in tables III and IV. The most striking result is the high sugar content of the lyophilized samples, although the higher contents of niacin and riboflavin and the lower contents of pyridoxin and starch in the same samples are noteworthy. The differences in starch and sugar can be accounted for by anabolism which continues during the air drying but is stopped promptly by lyophilizing. The effect of drying with heat as compared to air drying is shown in table V. High-moisture corn dried at high temperatures contains relatively high sugar and low starch. It seems that high temperatures stop anabolism as effectively as lyophilization. High

TABLE IV

VITAMIN CONTENT OF IMMATURE CORN GRAIN DRIED IN DIFFERENT WAYS.

Method of sampling	Initial moisture	Niacin	Pantothenic acid	Biotin	Riboflavin (B_2)	Pyridoxin (B_6)
	%	μg./gm.	μg./gm.	μg./gm.	μg./gm.	μg./gm.
Air-dried	68.5	27.7	14.2	.142	1.2	8.74
Lyophilized	69.0	58.3	14.4	.139	2.7	4.27
Lyophilized and oven-dried*	69.0	50.7	10.8	.124	3.0	4.15

*Samples were lyophilized and oven-dried at 100° C for 48 hours.

temperatures also may have reduced respiratory losses of sugars. The differences in the sugar and starch contents of samples dried at various temperatures were very small in the sample of grain having $26.3 \pm 2.3\%$ moisture. Somewhere between the limits of 40 and 24% moisture, the grain reached a stage of maturity beyond which the manner of drying no longer affected the sugar-starch system.

A significant correlation was found between total sugar and the drying temperature ($\gamma = 0.629$; required for significance at 1% level $\gamma = \pm 0.525$),

TABLE V

COMPOSITION OF CORN GRAIN WITH HEATED AIR COMPARED WITH CORN OF THE SAME KIND AND STAGE OF MATURITY DRIED WITH UNHEATED AIR. DATA ARE CALCULATED ON A MOISTURE-FREE BASIS.

Initial moisture range	Ash		Protein		Oil		Total sugar		Starch	
	Found	Difference	Found	Difference	Found	Difference	Found	Difference	Found	Difference
%	%	%	%	%	%	%	%	%	%	%
Dried at high temperature										
75.5 ± 0.2	2.83	+ 0.28	14.1	− 0.7	3.5	+ 1.1	11.64	+ 9.4	52.4	− 8.6
67.2 ± 1.8	2.64	+ 0.45	13.4	+ 0.6	3.7	+ 1.0	10.56	+ 8.45	56.6	− 8.4
51.4 ± 0.0	1.80	− 0.09	11.1	+ 0.5	5.2	+ 0.7	5.26	+ 2.96	66.3	− 3.3
46.4 ± 1.5	1.63	− 0.11	10.8	− 0.2	4.9	+ 0.4	4.92	+ 2.80	67.7	− 1.8
41.5 ± 2.7	1.53	− 0.05	9.6	− 1.4	4.8	+ 0.5	4.36	+ 2.55	70.4	0.0
40.3 ± 3.0	1.37	− 0.21	10.2	− 0.8	4.9	+ 0.6	3.55	+ 1.74	71.2	+ 0.8
26.3 ± 2.3	1.37	− 0.03	9.6	+ 0.3	4.8	+ 0.2	2.14	+ 0.17	73.3	− 0.4
Dried at medium temperature										
75.5 ± 0.2	3.21	+ 0.66	15.2	+ 0.4	3.8	+ 1.4	8.20	+ 5.56	55.3	− 5.7
67.2 ± 1.8	2.44	+ 0.25	12.8	0.0	3.5	+ 0.8	6.07	+ 3.96	64.0	− 1.0
46.4 ± 1.5	1.77	+ 0.03	10.8	− 0.2	4.9	+ 0.4	4.33	+ 2.21	68.9	− 0.6
41.5 ± 2.7	1.59	+ 0.01	10.3	− 0.7	4.9	+ 0.6	3.23	+ 1.42	71.0	+ 0.6
40.3 ± 3.0	1.54	− 0.04	11.1	+ 0.1	5.0	+ 0.7	3.38	+ 1.57	69.9	− 0.5
26.3 ± 2.3	1.44	+ 0.04	9.9	+ 0.6	4.9	+ 0.3	2.25	+ 0.28	72.2	− 1.5
Dried at low temperature										
67.2 ± 1.8	2.41	+ 0.03	12.4	− 0.4	3.4	+ 0.7	3.04	+ 0.93	66.0	+ 1.0
46.4 ± 1.5	1.76	+ 0.02	10.4	− 0.6	5.0	+ 0.5	3.21	+ 1.09	69.4	− 0.1
41.5 ± 2.7	1.52	− 0.06	10.2	− 0.8	5.0	+ 0.7	2.42	+ 0.61	71.8	+ 1.4
40.3 ± 3.0	1.56	− 0.02	10.2	− 0.8	4.9	+ 0.6	2.83	+ 1.02	71.1	+ 0.7
26.3 ± 2.3	1.41	+ 0.01	9.8	+ 0.5	4.9	+ 0.3	2.37	+ 0.40	73.7	0.0
Necessary for significance, t = 1%	0.124		0.850		0.187		1.73		1.868	

while no correlation existed between sugar content and drying time or humidity. Reducing sugar and total sugar were both high in samples dried at high temperatures.

No significant changes were found in protein ($N \times 6.25$) and ash except when corn with an initial moisture above 40% was dried at the high temperature. Percentages of oil were consistently higher in all samples of corn dried artificially than in comparable samples dried at room temperature. Rapid drying may have been responsible for this result by reducing respiratory losses. This is suggested by the fact that the very immature corn which was dried quickly showed the largest amount of oil compared with

the control. Protein was the only constituent which was practically constant in amount at all moisture levels.

Significant changes in vitamin contents caused by artificial dryings are shown in table VI. Of the vitamins assayed, niacin showed the greatest change due to drying conditions. Differences in biotin and carotene contents (not included in table VI) were not statistically significant. Differences significant at the 1% probability level were found in niacin, pantothenic acid, riboflavin, and pyridoxin. Pantothenic acid and pyridoxin decreased while riboflavin and niacin increased with high drying temperatures.

TABLE VI

COMPOSITION OF CORN GRAIN DRIED WITH HEATED AIR COMPARED WITH CORN OF THE SAME KIND AND STAGE OF MATURITY DRIED WITH UNHEATED AIR. DATA ARE CALCULATED ON A MOISTURE-FREE BASIS.

Initial moisture range	Niacin		Pantothenic acid		Riboflavin (B_2)		Pyridoxin (B_6)	
	Found	Difference	Found	Difference	Found	Difference	Found	Difference
%	μg./gm.	μg./gm.	μg./gm.	μg./gm.	μg./gm.	μg./gm.	μg./gm.	μg./gm.
Dried at high temperature								
75.5 ± 0.2	60.2	+ 21.9	9.5	− 1.8	2.8	+ 1.6	3.30	− 5.44
67.2 ± 1.8	55.9	+ 28.2	8.9	− 5.3	3.5	+ 2.3	3.58	− 5.76
51.4 ± 0.0	33.6	+ 9.4	7.3	− 5.1	2.0	+ 0.7	5.81	− 5.59
46.4 ± 1.5	29.1	+ 5.4	6.2	− 1.1	1.3	+ 0.1	4.70	− 1.9
41.5 ± 2.7	28.1	+ 4.9	8.1	− 1.5	1.4	− 0.7	4.61	− 1.22
40.3 ± 3.0	23.6	+ 0.4	6.2	− 3.4	1.2	− 0.9	4.94	− 0.89
26.3 ± 2.3	26.4	− 0.2	6.7	+ 1.1	1.2	− 0.3	5.62	− 1.16
Dried at medium temperature								
75.5 ± 0.2	52.1	+ 13.8	12.3	+ 1.0	2.3	+ 1.1	4.30	− 4.44
67.2 ± 1.8	39.2	+ 11.5	11.3	− 2.9	1.7	+ 0.5	4.34	− 5.00
46.4 ± 1.5	28.1	+ 4.4	9.8	+ 2.5	1.3	+ 0.1	5.67	− 0.93
41.5 ± 2.7	22.6	+ 0.6	7.9	− 1.7	1.9	− 0.2	6.90	+ 0.30
40.3 ± 3.0	20.9	+ 2.3	7.3	− 2.3	1.2	− 0.9	4.68	− 0.08
26.3 ± 2.3	25.4	− 1.2	8.5	+ 2.9	1.2	− 0.3	6.15	− 0.63
Dried at low temperature								
67.2 ± 1.8	24.2	− 3.5	13.0	− 1.2	1.9	+ 0.7	6.87	− 2.47
46.4 ± 1.5	22.6	− 1.1	6.9	− 0.4	1.2	0.0	5.93	− 0.67
41.5 ± 2.7	20.4	− 2.8	7.4	− 2.2	3.1	+ 1.0	7.59	+ 1.76
40.3 ± 3.0	19.8	− 3.4	8.2	− 1.4	2.8	+ 0.7	5.21	− 0.62
26.3 ± 2.3	26.0	− 0.6	6.2	+ 0.6	0.8	− 0.7	6.83	+ 0.05
Necessary for significance, t = 1%	3.925		1.331		0.50		1.780	

It is noted that pantothenic acid and riboflavin were the only constituents which showed significant differences when the corn contained less than 40% initial moisture.

The per cent. recovery of starch in processing artificially dried corn is shown in table VII. Both initial moisture content and drying temperature affected the processing of the dried corn for starch. The effect of drying temperature was apparent only in the high and medium moisture ranges. In general, however, the higher the initial moisture content and the higher the drying temperature, the lower was the percentage of starch recovered. The other processing data showed no clearly defined trends. Paste viscos-

ity of the recovered starch, as shown in table VII, was inversely related to the temperature at which the corn had been dried.

The viability of the corn, as indicated by the 2,3,5-triphenyltetrazolium chloride color test, was roughly proportional to the viscosity of the recovered starch. Correlation of the results of the test with processing data was less apparent, presumably because factors other than temperature, such as velocity and relative humidity of the drying air, affect the ease of processing of the dried corn.

TABLE VII

PROCESSING DATA ON ARTIFICIALLY DRIED CORN SAMPLES.

	Initial moisture range (in per cent.)											
	65.4 to 69.0				45.0 to 53.9				24.0 to 28.7			
Drying temperature, °F	180	130	110	75	180	130	110	75	180	130	110	75
% Starch recovery	57	60	72	79	65	67	69	75	71	73	68	71
Maximum viscosity of starch, gm. cm.	75	108	95	108	35	128	103	116	70	98	105	108
% Protein in starch	0.31	0.38	0.50	0.25	0.31	0.38	0.50	0.38	0.25	0.25	0.44	0.50
% Oil in starch	0.07	0.09	0.07	0.03	0.07	0.05	0.06	0.03	0.04	0.04	0.07	0.07
% Starch in fiber	39.1	44.2	36.4	26.0	48.6	43.8	47.3	35.0	42.4	40.5	44.1	41.5
% Protein in fiber	19.6	16.4	15.3	15.1	13.3	14.1	12.9	13.1	12.4	12.4	12.0	11.5
% Starch in gluten	57.0	49.9	36.4	43.3	48.2	44.1	48.9	47.6	50.4	42.6	53.5	51.6
% Oil in gluten	7.8	9.5	10.6	6.4	10.3	9.7	9.5	8.0	9.1	10.0	8.1	8.0
% Viability of grain	0	1	25	88	0	23	43	98	0	33	98	94

Summary

Corn varying from 24 to 75% initial moisture content was artificially dried at high (181.2° F), medium (129° F), and low (109.8° F) temperatures with controlled humidity and air velocity. Controls were ears air-dried at room temperatures and ears frozen in the field with dry ice and lyophilized in the laboratory.

Inbreds gave a differential response to the treatments in field performance as measured by yield of grain. Artificially dried, immature corn was more brittle than air-dried corn. Lyophilized samples contained more sugar and niacin, but less starch than air-dried corn.

Significant differences in niacin, pantothenic acid, riboflavin, pyridoxin, total sugar, and starch content were found when corn of high moisture content was dried at temperatures of 180 and 130° F. However, artificial drying was associated with very small differences in these constituents when the corn at harvest contained less than 40% initial moisture.

Corn samples with initial moisture content of 65.4 to 69.0% gave, upon processing, lower recovery of starch and concomitantly higher amounts of

starch in fiber and protein fractions than did the air-dried controls. Results at lower moisture ranges were inconsistent but revealed few differences due to drying with heat. The amount of protein in the fiber fraction decreased as corn advanced toward maturity, while starch in gluten and fiber fractions increased. The viability of the corn was roughly proportional to the viscosity of the recovered starch.

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EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON MERISTEMATIC TISSUES OF CORN[1]

DAVID W. STANIFORTH

(WITH FOUR FIGURES)

Received April 24, 1952

In recent years the use of 2,4-dichlorophenoxyacetic acid (2,4-D) as a selective herbicide has become increasingly important in the production of small grains and corn. Yield reductions due to direct injury to the crops are usually small. However, severe yield reductions have occurred occasionally when 2,4-D was used at herbicidal dosages during certain stages of crop growth. Corn plants sprayed with 2,4-D may develop very brittle stems which may be broken off by wind, resulting in a reduction in stand and yield. In small grains the yield reductions are largely due to various abnormalities in the growth of the spike which result in fewer kernels of grain (**3**).

A number of investigators (**1, 3, 4, 5, 6, 7, 10, 11**) have reported varying abnormalities in plant growth and yield reductions resulting from the application of 2,4-D to spring-seeded wheat, oats and barley at various stages of growth. Yield reductions resulting from applications of 2,4-D are commonly associated with two critical periods in the growth of barley and wheat: (a) an early seedling period when the plants are one to five inches tall and (b) a later period extending from the early boot stage to a few days before heading. A similar though less distinct pattern has been observed in oats.

ANDERSEN and HERMANSEN (**1**) found that the occurrence of spike abnormalities in oats, wheat and barley depended on the stage of development of the various plant parts at the time of treatment with 2,4-D. From this they concluded that the effect of the 2,4-D was on the differentiation of cells forming the first rudiments of a plant organ. DERSCHEID (**3**) working with oats and barley, showed that stage of growth of the crop plant at the time of application of 2,4-D was the most important single factor affecting subsequent injury as measured by yield of grain. He found that treatments of barley seedlings with 2,4-D reduced yields severely whenever the treatments were made during tiller bud differentiation and during floral initiation. The yield reductions were manifest by a reduction in numbers of tillers and spikes, and by a reduction in seeds per spike. When floral initiation was rapid, the yield reduction was severe only at one or two dates of treatment, but when the floral initiation was slow, the yield reductions were less severe at any one time, but reductions were found in more treatments. Floral initiation in the tillers occurred later than in the central culm of the plants.

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Thus yield reductions were evident at several dates of treatment with 2,4-D after floral initiation had occurred in the central culm. When floral initiation was rapid, there was a short period between floral initiation in the central culms and tillers during which treatment with 2,4-D did not cause yield reductions. When floral initiation was slow, there was considerable overlapping of initiation in central culms and tillers; and yield reductions occurred throughout this period. DERSCHEID (**3**) also found that yield reductions from spray treatments with 2,4-D made during the second susceptible period, before and during heading, were not so clear cut as for the earlier seedling period. However, he postulated that embryo sac development and gamete differentiation were inhibited, thus reducing the number of seeds formed and the yield.

FRIESEN and OLSON (**5**) treated barley plants grown in the greenhouse, with 2,4-D and found two periods during which the plants were very susceptible to injury. The first period occurred during the time of spike differentiation in the growing point; the second period began with anther and stigma differentiation and continued through the growth stages of the flowering parts. ROSSMAN and STANIFORTH (**9**) noted the occurrence of barren tassels in certain inbred lines of corn which had been treated with 2,4-D. They also reported severe reduction in seed set in corn following heavy applications of 2,4-D. RODGERS (**8**), working with inbreds and single crosses of corn, found that the stage of development of the corn plant was an important factor in the degree of injury resulting from treatment with 2,4-D.

The present work was done in the season of 1948 and has been reported in abstract form (**12**). The work was designed to determine the effects of 2,4-D when applied to plants at specific stages of morphological development. The corn plant was utilized because of its economic importance and its morphological suitability for the study.

Methods

Three inbred lines of corn, M14, W22 and WF9, grown in nursery rows of 50 plants were used to study the effect of applications of 2,4-D on the development of tassels. Plants of the inbred lines were dissected and examined under a dissecting microscope with a magnification of 30 diameters to determine the time of floral initiation. The first application of 2,4-D was made on June 24, approximately 30 days after seeding. At this time approximately two thirds of the 10 or 12 plants examined from each line showed the first microscopic evidence of tassel initiation. Further applications were made to other plants on June 30 and July 5. The application of 2,4-D on each date was one half pound acid equivalent per acre of the sodium salt in 120 gallons of water, with .5% by weight of Dreft (**13**) added to the spray solutions. The solution was applied to the aerial portions of the plants with a knapsack sprayer.

Tassel primordia from each of 10 plants per inbred line were obtained at the time of spraying and at intervals after spraying, and preserved for

later study. The mature tassels of the remaining plants were later collected and the number of lateral branches per tassel and the number of barren tassels were determined.

Additional nursery rows of the single cross hybrid WF9 × 38–11 were grown for use in studies of the effects of 2,4-D on the development of ear shoots and seed set. The treatments employed the sodium salt of 2,4-D at a concentration of 1600 p.p.m. in water containing .5% by weight of Dreft, applied with a knapsack sprayer to thoroughly wet the leaves.

The effects of 2,4-D on seed set on the ears of corn were determined by making applications before and after emergence of the silks. The developing ears and silks were protected by bags while making the spray applica-

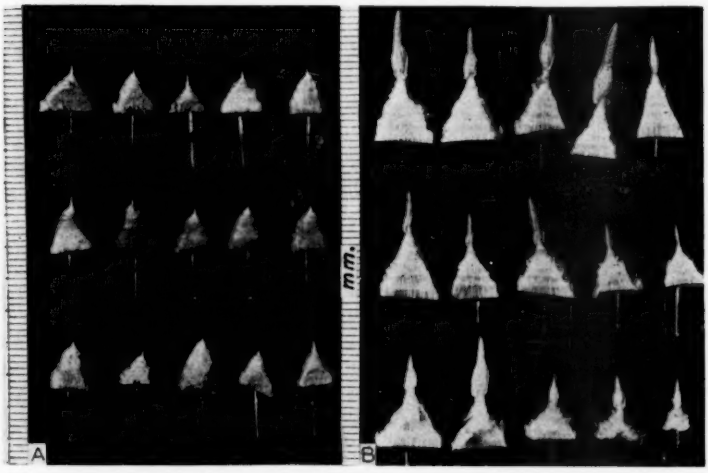

FIG. 1. A. Tassel primordia showing the stage of development of inbred lines at the time of treatment with 2,4-D on June 24. Top to bottom: M14, WF9 and W22. B. Developing tassels of W22 sampled July 5. Top to bottom: no spray, 2,4-D applied June 24, and 2,4-D applied June 30.

tions. The bags were left on the ears for varying periods when it was desired to delay pollination beyond the treatment date. No hand pollinations were made, but an abundance of pollen was available at all times. Treatments consisted of spraying at varying periods in relation to emergence of the silks and to exposure of the silks to pollination. On plants sprayed July 27, the silks emerged July 28 and the bags were removed for pollination on July 30. On plants sprayed July 27, the silks emerged July 29 and were pollinated August 2. On plants sprayed July 31, the silks emerged July 29 and were pollinated July 31. On plants sprayed August 5, the silks emerged July 29 and were pollinated August 5. On plants sprayed July 27, the silks emerged July 31 and were pollinated August 9. Check material for each treatment was handled in the same manner, except the

TABLE I

LATERAL BRANCHES PER TASSEL AND THE PERCENTAGE OF PLANTS WHICH DID NOT PRODUCE A TASSEL AS A RESULT OF TREATMENT WITH 2,4-D.

Inbred	Date of treatment	Number of plants examined	Average number of lateral branches per tassel	Percentage of plants without a tassel
M14	June 24	26	5.3	15.4
	June 30	58	10.0	8.6
	July 5	69	10.0	14.5
	No treatment	32	9.4	0
W22	June 24	36	4.1	33.3
	June 30	58	12.0	19.0
	July 5	45	12.2	17.8
	No treatment	66	13.0	0
WF9	June 24	79	6.2	1.3
	June 30	86	11.4	0
	July 5	30	13.3	6.7
	No treatment	44	13.6	0

treatment with 2,4-D was omitted. In another series of treatments, plants were sprayed 0, 2, 4 and 8 days after silking. No protective bags were used in this test, and the silks were exposed to pollen at all times after emergence. The number of plants in each group ranged from 15 to 25. Ears were collected at maturity, when observations were made and representative photographs obtained.

Experimental results

Applications of 2,4-D to the foliage of the three inbred lines, at or near the time of visible tassel initiation, reduced the number of lateral branches

FIG. 2. Mature tassels from W22 plants sprayed June 24. Tassel from an untreated plant on the left.

per tassel and resulted in an increase in the number of plants which did not produce a tassel. Figure 1 shows developing tassel primordia of the three inbred lines at the time of the first treatment with 2,4-D. Table I presents data on the numbers of lateral branches on mature tassels from treated and untreated plants. The treatments which were made 6 and 11 days after visible tassel initiation did not cause any significant change in the number of lateral branches per tassel but did result in some plants not producing a tassel. These later treatments caused some delay in the growth of tassels, as shown in figure 1. Plants sprayed June 30 (lower row of tassel primordia) showed very few visible morphological deficiencies but their size was reduced. Figure 2 illustrates the inhibition of tassel development, as shown at maturity, on plants of W22 treated with 2,4-D at the time when tassel initiation was visible.

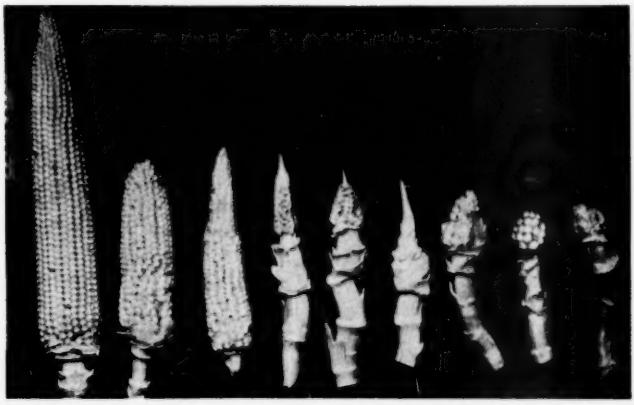

FIG. 3. Inhibition of ear development by 2,4-D spray applied just before emergence of the tassel. Ear from untreated plant on the left. Photographed one month after spraying.

Examination of many mature and developing tassels indicated that inhibition of the tassel development was not uniform for any particular date of treatment with 2,4-D. Similarly the degree of development visible in the untreated material varied considerably, so that all the plants were not in the same stage of tassel development at any one date of treatment. The number of lateral branches per tassel varied widely within a given treatment. A few individual plants from each inbred line which were sprayed on June 30 or July 5 had fewer lateral branches than the mean value for those plants sprayed on June 24. This variability in response indicates that the susceptible period is relatively short. Apparently the effect of the 2,4-D on the meristematic tissues of the developing tassel ranges from complete tassel failure through inhibition of lateral branches to little if any injury, depending upon stage of development of the tassel at the time of treatment. Developing tassels showed approximately the same degree of

inhibition one or two weeks after treatment as they did in the mature tassels. The inhibitory action of the 2,4-D was then apparently permanent on the particular meristematic tissue affected and there was no recovery or differentiation of new tassel branches.

Inhibition of the development of ear shoots was noted in plots of single cross WF9 × 38–11 which were treated at the rate of one half pound of the sodium salt of 2,4-D with .5% by weight of Dreft added to the spray solution. The spray treatments, made just as the tassels were beginning to emerge, caused almost complete suppression of the ear shoots in some plants. In other plants, the ears were of varying size and number per plant. These responses indicate a inhibition of the differentiating cells of the early rudiments of the organ of the ear shoot. The inhibition ranged from complete suppression of all ear development on some plants, through severe

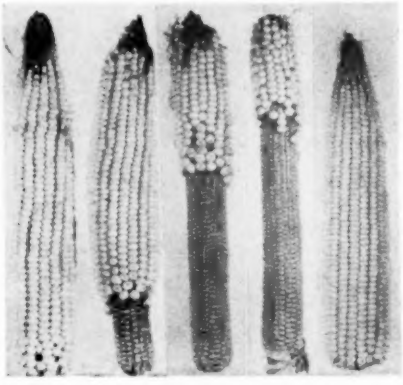

FIG. 4. Left to right, ears from corn plants sprayed 0, 1, 2 and 4 days before emergence of silks; ear from untreated plant on the right.

stunting of several ears on one plant, to only a slight stunting of a single ear on other plants. All the untreated plants produced a single ear. Figure 3 illustrates the several types of inhibition produced.

Spraying the corn plants one, two, or four days before silking, resulted in a severe inhibition of seed set in the lower portion of the ear, as shown in figure 4. The band of seed around the top of the ear was typical of all the ears examined when the treatment with 2,4-D had been made one, two, or four days prior to silking. In some ears the barren area was less than one half the area of the total ear, but it was always confined to the lower part of the ear. None of the ears examined, from more than 100 plants which were sprayed one to four days before silking, showed any variation from the pattern of seed set illustrated in figure 4. Delaying pollination for nine days after silk emergence, on plants which were sprayed before silking, did not reduce the inhibition of seed set. Since the delay in pollination did

not reduce the effect of the 2,4-D, it is probable that the effect is on the maternal tissue and not on the pollen which falls on the silk. This conclusion assumes that the action of 2,4-D is rapid, which seems to be borne out by other experiments in this study. Plants which silked July 29 and were sprayed July 31 and exposed to pollination immediately after spraying showed no evidence of reduced seed set and did not differ visibly from ears which were not sprayed. Plants sprayed 0, 2, 4, and 8 days after silking and exposed to pollen, produced normal ears at all times. The inhibitory action of the 2,4-D on seed set was evident only when the 2,4-D was applied at least one day prior to the emergence of silks. When the treatment with 2,4-D, silk emergence and pollination all occurred on the same day, or when treatments with 2,4-D were made after silk emergence and pollination there was no inhibition of seed set.

Discussion

Treatments with 2,4-D at certain critical growth stages resulted in a severe inhibitory effect on the development of the inflorescences of the corn plant. These critical growth stages were of relatively short duration and apparently occurred during meristematic differentiation of the reproductive rudiments of the plant.

The inhibition of spike (tassel) development resulting from treatments with 2,4-D at the time of early initiation, has been duplicated in later investigations by other workers employing barley (**1, 3, 5**). The spike abnormalities caused by the treatments probably are the result of the same inhibition pattern which produced the abnormal tassel development found in this study. In all instances the inhibition of barley spikes and corn tassels was evident only when the growth substance was applied during the period of initiation of the inflorescences. Evidence to date indicates that the period of susceptibility is short for any differentiating tissue. Considerable variation was observed in the degree of tassel inhibition among the corn plants treated at a given date, indicating that some of the plants were not in the susceptible stage of growth at the time of the treatment.

The inhibition of seed set resulting from pre-silking treatments with 2,4-D exhibits a distinct parallel with the results which have been observed with barley. DERSCHEID (**3**) has described a susceptible period for barley, occurring approximately during anthesis. Spray treatments with 2,4-D during this pre-heading period caused yield reductions by reducing the number of seeds formed in the spike. ANDERSEN and HERMANSEN (**1**) found a similar susceptible period and postulated that it occurred about the time that sex-cells were being formed. FRIESEN and OLSON (**5**) have also described this susceptible growth period in barley.

The results obtained with treatments with 2,4-D in this study showed a severe inhibition of seed set when the 2,4-D was applied four days before the silks emerged. Treatments at shorter intervals before silking resulted in the inhibition of seed set on a smaller portion of the ear. The pistillate

flowers develop from the base toward the tip of the ear (**2**). If the 2,4-D stimulus was carried first to the lower flowers this would explain in part the pattern of inhibition shown in figure 4. The inhibitory action of the 2,4-D apparently is effective only at a particular stage of development, perhaps at a stage very close to the actual emergence of silks, since plants treated one day before silking showed inhibition only on the lower part of the ear. However plants sprayed four days before silking show almost complete inhibition of seed set as seen in figure 4. The pattern of increased inhibition of seed set as the interval between spraying and silking is lengthened has not been explained.

The effects of 2,4-D in reducing the development of tassels, in suppressing the development of ear shoots and in inhibiting seed set on developing ears, show a similarity with respect to the kind of cells affected. Considering the three types of injury together, it seems probable that the inhibitory effect occurs during specific meristematic differentiations. The 2,4-D prevents the formation of flowering spikes, either partially or completely, depending upon the stage of development. It has apparently little, if any, effect on the growth of tassels once they are differentiated. Similarly the 2,4-D interferes with the initiation of pistillate flowers, and when applied at pre-silking it may prevent the formation of macrogametes. It appears to have little or no effect on the growth of seeds when applied after pollination.

Summary

Plants of corn inbreds M14, W22 and WF9 were sprayed with 2,4-D, 0, 6 and 11 days after the first visible signs of tassel initiation in the growing points. Plants of the single-cross hybrid, WF9 × 38–11 were sprayed with 2,4-D at the time of tassel emergence, or at various times in relation to silk emergence.

The treatment at the time of tassel initiation caused severe inhibition of tassel development. The later treatments caused much less inhibitory effect. Applications of 2,4-D one to four days before silk emergence caused severe inhibition of seed set on the developing ear. Applications after silking had no effect on ear development. Treatment with 2,4-D at the time of tassel emergence resulted in the inhibition of ear shoots which were developing. Treatments with 2,4-D at critical stages of growth resulted in a severe inhibitory effect on the development of the inflorescences of the corn plant. The types of injury produced indicate that the inhibitory effects occur during meristematic differentiation.

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BRIEF PAPERS

SOME EFFECTS OF ZINC ON SEVERAL SPECIES OF GOSSYPIUM L.

LAMAR C. BROWN[1] AND CHARLES C. WILSON

(WITH TWO FIGURES)

Received December 6, 1951

The determination of the factors which are operative among the plants of a genus in the establishment of specific differences constitutes one of the interesting problems of biology. There are numerous approaches to this problem, but one approach which seemed particularly germane to the writers concerned the effect of zinc upon the general growth and metabolism of plants.

Although zinc has been established for many years as an essential element in plant nutrition, it has only been fairly recently that sufficient evidence has been accumulated through the work of SKOOG (**2**), TSUI (**5**), and others to establish an hypothesis concerning its mode of action. In general the evidence seems to indicate that, in some manner, there is a relation between zinc and auxin production and/or destruction within the plant. Regardless of its precise method of action, there seems little question that in the absence of zinc the level of auxin is depressed. Not only has this fact been confirmed quantitatively through the work of SKOOG (**2**) and of TSUI (**5**), but the symptoms of zinc deficiency reported by many workers are all congruent with the concept of a low level of the growth hormone.

Data have been obtained (**6**) which clearly indicate rather important inheritable physiological differences among at least four species with respect to effect of temperature on growth. It has also been reported by STEPHENS (**3**) that reciprocal grafts between various cotton species produced differential effects. It is considered that some of these effects might be associated with various levels of auxin production or sensitivity between the various species. This hypothesis might be tested by controlling the level of zinc, hence of auxin, in the plant and observing the general growth responses for differential effects. An experiment establishing the need for zinc by *Gossypium hirsutum* indicates the probable requirement of all species for this nutrient element (**7**).

The species of cotton selected for this experiment were two American tetraploid (n = 26) species, *Gossypium barbadense* L. (Sea Island–Ex. Jenkins), and *Gossypium hirsutum* L. (Coker 100-W); and one Asiatic diploid (n = 13) species, *Gossypium arboreum* L. (race—sinense). Observations were made on plants growing in an unshaded greenhouse. Plants were grown in two-liter Pyrex beakers which had previously been painted on the

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outside with a coat of black asphaltum followed by a layer of aluminum paint. To permit drainage a small hole was bored in the bottom of each beaker, and a piece of Pyrex wool placed over the hole. Fine quartz sand was washed with concentrated sulphuric acid and then rinsed with Pyrex-distilled water. The sand was then placed in the beakers and washed with a 1:1 solution of hydrochloric acid followed by a thorough rinsing with distilled water. Cardboard tops, impregnated with paraffin and punctured with several small holes, were used as covers for the beakers.

Nutrient solutions were prepared and purified separately, 5 liters at a time, in 6-liter flasks. Major nutrients were prepared using the methods of ARNON (**1**) and STOUT and ARNON (**4**) in which molar solutions of salts were used. The nutrient solutions and distilled water used in the experiment were tested frequently by the dithizone test, as described by STOUT and ARNON (**4**), and found to contain less than one part in ten billion of trace

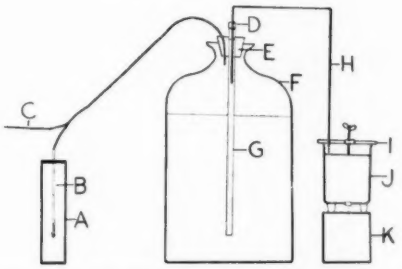

FIG. 1. Apparatus used to transfer the nutrient solution to the plants. A. Pressure regulator. B. Glass tubing. C. Air inlet. D. Cork stopper. E. Rubber stopper. F. 12-gallon bottle. G. 1-cm. Pyrex tube. H. Capillary tube. I. Cardboard top. J. Beaker containing sand. K. Stoneware crock.

metals. A trace supplement, which consisted of boron, 0.5 p.p.m.; copper, 0.02 p.p.m.; manganese, 0.5 p.p.m.; and a 0.5% iron solution, was used. Concentrations of zinc of 0.000 p.p.m., 0.001 p.p.m., and 0.100 p.p.m. were used.

Nutrient solutions were stored in 12-gallon Pyrex bottles which had been cleaned with hydrochloric acid and rinsed with trace-free distilled water. The bottles were painted on the outside with black asphaltum followed by a layer of aluminum paint. Each concentration of zinc was added to separate 12-gallon Pyrex bottles so that there was a total of eight bottles for each concentration of zinc. Four beakers, each containing a single species of cotton, were arranged around each nutrient bottle. In order to maintain a constant flow of nutrient solution into the beakers, the setup in figure 1 was constructed. The height of the nutrient solution in the Pyrex tubing could be maintained by adjustment of the glass tube in the pressure regulator. The capillary tubing was calibrated, as to rate of flow, by adjusting

the capillary tubing up or down in the Pyrex tubes and counting the number of drops.

A plant of each species from each concentration of zinc, chosen at random, was harvested every two weeks beginning August 28, when the plants were three weeks of age, and continuing through December 4, 1950. Leaves and fruits were removed from the stem, and fresh weights of leaves, fruits, stems, and roots were taken. The leaves were traced on bond paper and leaf areas later computed by the use of a planimeter. Counts were made of the number of squares, number of fruits (inflorescences after anthesis were counted as fruits; inflorescences prior to anthesis were counted as squares), and the number of leaves. Drying was accomplished in a forced-draft oven for a period of one half hour at 105° C followed by a 72 hour period at 60° C. A record of symptoms of zinc deficiency and interspecific differences between species was made at intervals between harvests and at the time of harvest. For simplicity the 0.000 p.p.m. concentration of zinc is designated as minus-zinc, the 0.001 p.p.m. concentration as low-zinc, and the 0.1 p.p.m. concentration as controls.

The control plants of all species of cotton were healthy throughout the experiment, and did not present any evidence of deficiency symptoms at any time during this study. The first zinc deficiency symptoms, in the form of necrotic spots, occurred on the cotyledons of the minus-zinc plants at the age of 16 days. Zinc deficiency symptoms were more pronounced when the plants of the minus-zinc series had reached an age of three weeks. At this time the minus-zinc plants of *G. hirsutum* and *G. barbadense* were somewhat dwarfed, and the leaves were slightly chlorotic and somewhat hardened. *G. arboreum* plants were not dwarfed; however, the leaves exhibited a trace of chlorosis. The roots of the American species in the minus-zinc series were stunted in growth; while roots of the minus-zinc plants of *G. arboreum* were seemingly healthy. The roots of the low-zinc series did not exhibit stunting at this time, but were not as vigorous as the controls.

Zinc deficiency symptoms were quite evident at the age of five weeks. Chlorosis was very distinct. Tinges of yellow were present between the veins, and some small white spots were scattered over the surface of the leaves. Forms of chlorosis were evident in the leaves of the low-zinc plants at this time, but not to the extent of that in the minus-zinc plants. The leaves continued to exhibit necrotic areas which varied in size and distribution on the surface of the leaves. These areas were more pronounced in the minus-zinc plants, and were larger and darker in color in the American species. These necrotic areas persisted throughout the remainder of the experiment. Zinc deficiency symptoms such as stunted growth, reduced leaf size, and shortened internodes were quite pronounced at this time. Striking differences in height between plants of the minus-zinc series and control plants could be clearly seen. The leaf area was greater in the American species than in the Asiatic species. Defoliation was evident during the fifth week of growth in the minus-zinc plants, and some defoliation occurred in

the low-zinc plants. The roots were stunted in all the zinc deficient plants, and some die-back was noted in the secondary roots of the minus-zinc series.

An inrolling of the margins of the leaves of the minus-zinc plants of the American species occurred when the plants were seven weeks old. This symptom was more pronounced in leaves of the minus-zinc plants but occurred in a less extreme form in the leaves of the low-zinc plants. The leaves of the minus-zinc plants were quite brittle by this time, and extreme chlorosis was evident in most of the minus-zinc plants. Leaves of minus-zinc plants of *G. hirsutum* presented the most extreme chlorosis in which large areas of the leaf were completely white. In some instances only a section of the leaf around the midrib retained its green color (fig. 2). The leaves of zinc deficient plants of *G. arboreum* did not exhibit this extreme type of chlorosis although chlorosis was very distinct. It was also noted that there was a greater accumulation of anthocyanin in the petioles of the

FIG. 2. Chlorosis in a minus-zinc leaf of *G. hirsutum* at age seven weeks.

American species; however, the amount of anthocyanin varied with plants in different concentrations of zinc. Die-back of the roots was quite evident in all the species and large amounts of secondary roots were dead. An inrolling of the leaves of minus-zinc plants of *G. arboreum* did not occur until the plants were 17 weeks old; however, many of the leaf tips curled inward at the age of seven weeks and in a short time the tisssue around the leaf tip had a burned appearance.

One of the common symptoms of zinc deficiency was the lack of square formation. Zinc deficient plants of *G. barbadense* did not produce any squares throughout the experiment, and only a few squares were formed on the zinc deficient plants of *G. arboreum* and *G. hirsutum*. Two of the minus and low-zinc plants of *G. hirsutum* produced a few squares, but these shed before anthesis. Four plants of the minus-zinc and low-zinc series of *G. arboreum* produced squares, but only two of the squares of the low-zinc series remained on the plant until the stage of anthesis, and these shed a short time thereafter.

The results of the various concentrations of zinc were most conspicuously evidenced in the growth in height of the various species of plants (table I). The results of the various concentrations of zinc are even more strikingly shown by expressing the total height of the control plants of each species as percentage of the total height of the minus-zinc plants of the same species. Thus *G. hirsutum* was 138%, *G. barbadense* 168%, and *G. arboreum* was 285%.

The results seem to offer rather striking confirmation of the hypothesis that various species of cotton plants would exhibit differential effects when grown under different levels of nutrient zinc, presumably through the effect of zinc on the auxin levels of the plants. Of all the effects on plant growth which have been ascribed to the action of auxin there is probably none on which there is more common agreement than its effect on growth in height. It is in this effect that these data seem to indicate the greatest difference between the response of the various species. The data of table I clearly indicate that the response of *G. arboreum* when compared to *G. hirsutum* at

TABLE I

HEIGHT OF *Gossypium barbadense* AND *Gossypium arboreum* EXPRESSED AS PER CENT. OF HEIGHT OF *Gossypium hirsutum* WHEN PLANTS WERE 17 WEEKS OLD.

Zinc concentration	*G. hirsutum*	*G. barbadense*	*G. arboreum*
p.p.m.			
.000	100	94	71
.001	100	100	98
.100	100	115	150

various levels of zinc is quite different. Normally, as indicated by the controls, arboreum is a much taller plant than hirsutum at maturity. However, in the absence of zinc, it only reached 71% of the height of the minus-zinc plants of hirsutum. As the concentration of zinc was increased the growth of arboreum increased proportionally more than that of hirsutum. Finally when zinc was added in what must be assumed was optimum quantity, the plants of arboreum were more than 50% taller than those of hirsutum.

The results showing the growth of the controls of each species compared to the minus-zinc plants of the same species indicate in an even more striking manner the differential effect of zinc upon the various species. The control plants of *G. hirsutum* were 38% taller than the minus-zinc plants, a relatively small increase while the control plants of *G. arboreum* were almost three times taller than the minus-zinc plants.

There are several alternate hypotheses to account for this differential response. The results of Skoog (**2**) and those of Tsui (**5**) clearly indicate that in the absence of zinc there is a reduction in the auxin level. Tsui further showed that the effect may be more the result of auxin destruction than

of production. We may assume that in the plants of the experiments reported here, the auxin levels were reduced by the absence of zinc; but the various species were able to maintain different levels of auxin regardless of the absence of zinc. Thus the plants of *G. hirsutum* were not as greatly affected by the absence of zinc as were those of *G. barbadense* or *G. arboreum* and maintained a higher level of auxin. It is also possible that the levels of auxin in the plants of the various species were all reduced to approximately the same level but that in some way the plants of *G. hirsutum* were able to use what remained more effectively. It is not possible to infer from these data which hypothesis is correct. It does seem quite clear however that either the mode of action of auxin or the sensitivity to it does vary among the different species and does provide the basis for future investigations concerning the differences between the species.

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THE EFFECT OF DIFFERENT TISSUE PRETREATMENTS ON THE PHYSICO-CHEMICAL PROPERTIES OF EXPRESSED PLANT SAP

G. K. Voigt

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The information accumulated by plant physiologists reveals more and more clearly the importance of the physico-chemical properties of plant sap in the metabolic activities and resistance of plants to adverse environmental factors. Although pretreatment of plant tissue has received the attention of several workers (**4, 7, 11, 12**), no systematic comparison of the methods employed has been made in regard to coniferous needles. The present study was conducted to investigate the effect of different expression pressures, various methods of tissue pretreatment and time of sampling on the yield and physico-chemical properties of plant sap expressed from coniferous foliage.

Needles from the current season's growth were collected in June from a plantation of 15-year-old red pines, *Pinus resinosa* Ait. Most of the samples were sealed in air-tight plastic bags, frozen with solid CO_2, and taken to the laboratory for analysis. The sap was expressed by means of the Carver Laboratory hydraulic press and cage equipment. In most cases, a pressure on the tissue of 2,080 lbs. was used. This procedure removed about 60% of the total moisture from the tissue. The expressed sap was subjected to centrifuging for 10 minutes at approximately 1000 times gravity, but no solid material was separated and subsequently centrifuging was abandoned. The volume of the expressed sap was recorded and the freezing point depression, conductance, and content of total solids were determined in as short a time as possible. The sap was frozen and stored in a freezer at $-5°$ C for further analysis.

The freezing point depression was obtained by means of the Drucker-Burian cryoscopic apparatus (**5**). The cell sap was undercooled one degree before being seeded with an ice crystal. The observed freezing point was corrected by using the formula and table of Harris and Gortner (**10**). The electrical resistance was determined by using a resistance bridge with a platinum conductance cell, manufactured by the Industrial Instrument Company. The temperature was controlled at 28° C by means of a water bath. The resistance of a sample of 10 ml. of sap was determined and converted to conductance (**9**). The total solid content of the sap was determined by the refractometer method of Gortner and Hoffman (**8**). The refractive indices were converted to per cent. total solids by the International Scale of Refractive Indices of Sucrose Solutions, and temperature corrections were made by means of the International Correction Tables (**1**).

The content of ash was determined by slowly evaporating 20 ml. of plant sap to dryness on an electric hot plate with subsequent heating in an electric muffle furnace at 450° C for 12 hours. After weighing, the ash was dis-

TABLE I

THE EFFECT OF DIFFERENT EXPRESSION PRESSURES UPON THE YIELDS AND PROPERTIES OF CELL SAP FROM NEEDLES OF RED PINE. THE TISSUE WAS AUTOCLAVED FOR 30 MINUTES AT 15 POUNDS PER SQUARE INCH.

Pressure on tissue	Yield	Conductance	Total solids	Freezing point depression
lbs./in.²	*ml./100 gm.*	*Mhos × 10³*	*%*	*° C*
520	23	4.62	13.2	− 1.230
1040	29	4.70	14.7	− 1.270
1560	37	4.69	14.2	− 1.249
2080	42	4.81	14.6	− 1.261

solved in HCl and diluted to 100 ml. in a volumetric flask. Aliquots of this solution were used for the determination of mineral nutrients. A similar procedure was followed in the case of plant tissue, except that a sample of one gram of tissue was ashed.

Total nitrogen in the tissue and soluble nitrogen in the plant sap were determined by the standard Kjeldahl procedure (**1**). The content of phosphorus was determined colorimetrically by an adaptation of the phosphovanadomolybdate method (**3**). The determination of potassium was made by means of the Perkin-Elmer flame photometer on an aliquot of 10 ml. of the solution of the ash which was evaporated to dryness and taken up in 50 ml. of extraction solution (**2**).

In a preliminary experiment, freshly collected tissue samples were placed in loosely covered Mason jars and autoclaved for 30 minutes at 15 pounds per square inch. The covered jars and their contents were removed and cooled to room temperature. Equal amounts of needle tissue were subjected to four different pressures in a hydraulic press. The expressed sap was collected and analyzed immediately. Another sample of needles was divided into four lots: one lot was diced into small pieces; a second lot was diced and autoclaved for 30 minutes at 15 pounds per square inch; a third lot was diced and steamed for 30 minutes; the fourth lot was diced and frozen with solid CO_2 for 12 hours. After the sap had been expressed and its volume recorded, the properties of the fluid were determined. The press cakes and a sample of unexpressed tissue were dried and ground for analysis.

TABLE II

EFFECT OF DIFFERENT PRETREATMENTS UPON THE YIELD AND PROPERTIES OF CELL SAP FROM NEEDLES OF RED PINE. EXPRESSION PRESSURE ON THE TISSUE WAS 2,080 POUNDS PER SQUARE INCH.

Treatment	Yield	Osmotic pressure	Total solids	Cond.	Ash	N	P	K
	ml./100 gm.	*atmos.*	*%*	*Mhos × 10³*	*mg./20 ml.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
Diced	25	4.46	6.9	2.49	42.1	265	34	350
Autoclaved 30 minutes at 15 lbs./in.²	36	15.35	15.3	4.70	128.0	265	240	1200
Steamed 30 minutes	35	15.76	15.3	4.87	134.9	280	270	1215
Frozen with solid CO_2 for 12 hours	32	16.66	16.0	4.94	136.6	275	217	1237

TABLE III

EFFECT OF DIFFERENT TREATMENTS ON THE CONTENT OF ASH AND ESSENTIAL NUTRIENTS IN PRESS CAKES AFTER EXPRESSION OF PLANT SAP.

Treatment	Ash	N	P	K
	%	%	%	%
Untreated needles	2.41	0.54	0.21	0.75
Expressed diced needles	2.33	0.52	0.19	0.70
Expressed autoclaved needles	2.03	0.50	0.16	0.53
Expressed steamed needles	1.87	0.51	0.17	0.42
Expressed frozen needles	1.93	0.49	0.16	0.40

The effect of the time of sampling was investigated by collecting tissue from the same red pine plantation at 8 A.M. and 4 P.M.

The results of expression pressure trials summarized in table I indicate that the amount of pressure has no significant effect on the properties of the plant sap. The yield, however, is increased markedly by the higher pressure. This fact may be of importance in studies in which the supply of tissue produced is limited.

The effects of different methods of pretreatment of tissue on the yield and physico-chemical properties of sap are shown in table II. These results are in agreement with the findings of DIXON and ATKINS (**6**) who concluded that unless the tissue is killed, retention of solutes by the living cell membranes leads to low values. Pretreatment by autoclaving or steaming gave practically the same analysis, but the values for the characteristics of sap expressed from frozen tissue were slightly higher. The content of soluble nitrogen, however, remained approximately the same for all treatments which indicates that this fraction is not coagulated by freezing or high temperature, since no decrease in soluble nitrogen was observed in the sap from tissue killed by freezing or heating. The results suggest that the use of solid CO_2 is most advantageous especially where field collection of tissues is necessary. If the samples are sealed in plastic bags and placed in an insulated container with sufficient solid CO_2, the tissue may be kept frozen as long as an adequate supply of solid CO_2 is maintained. This procedure insures minimum metabolic changes.

The chemical composition of the press cakes, given in table III, complements, to some degree, the composition of the cell sap.

The importance of uniform sampling technique is demonstrated by the

TABLE IV

EFFECTS OF TIME OF COLLECTION OF NEEDLE TISSUE ON YIELD AND PROPERTIES OF EXPRESSED CELL SAP (AVERAGE RESULTS).

Time of sampling	Yield	Osmotic pressure	Conductivity	Total solids
	ml./100 gm.	*Atmos.*	*Mhos* $\times 10^3$	%
8 A.M.	48	14.81	4.59	13.2
4 P.M.	31	16.74	4.96	16.8

data in table IV. Although the same trees were sampled, the foliage collected in the afternoon had been subjected to a longer period of transpiration and showed a higher sap concentration. It is also likely that the content of non-electrolytes was higher because of photosynthesis during the day since the value for osmotic pressure increased by 13% while the conductivity was only 8% greater.

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THE USE OF CHROMOTROPIC ACID FOR THE QUANTITATIVE DETERMINATION OF 2,4-DICHLOROPHENOXY-ACETIC ACID[1]

DUANE LETOURNEAU AND NORMAN KROG

(WITH TWO FIGURES)

Received April 7, 1952

FREED (**7**) described a qualitative test for 2,4-dichlorophenoxyacetic acid (2,4-D) based on the formation of a purple color developed when 2,4-D and chromotropic acid were heated in concentrated H_2SO_4. However, Freed was not successful in adapting this test for quantitative determinations. The present investigation was undertaken to develop a quantitative method for the determination of 2,4-D based on Freed's reaction and to obtain information concerning its specificity.

Technical grade, 2,4-D was recrystallized twice from benzene, M.P. 138–139. Chromotropic acid (Eastman practical grade) was recrystallized by the method of BRICKER and JOHNSON (**3**). The sulphuric acid was a C.P. reagent of sp. gr. 1.84. The sulphuric acid-chromotropic acid reagent was made by dissolving 400 mg. of chromotropic acid in 200 ml. of concentrated H_2SO_4. Standard 2,4-D solutions were prepared by dissolving 35 mg. of 2,4-D in sufficient benzene to make one liter and diluting with benzene to 2.5 to 35 γ/ml. One milliliter of the standard or unknown was pipetted into a cuvette (12 × 75 mm.) and evaporated by the microvacuum distillation method of JUDKINS (**8**) and 2 ml. of the sulphuric acid-chromotropic acid reagent were added to each cuvette. A blank with reagents was included. The cuvettes were heated in a glycerine bath at 150 to 155° C for two minutes. After cooling, the optical density of the solution was measured in a Coleman spectrophotometer at a wave length of 580 mμ. with the instrument set at 100% transmission with the reagent blank.

Preliminary experiments showed that the colored solutions resulting from this procedure gave the highest optical density or absorption at wave lengths of 580 mμ. (fig. 1), and therefore all measurements were made in this region.

An accurate control of the amount of chromotropic acid was found to be important for the successful application of the procedure (table I) and was accomplished by dissolving chromotropic acid in concentrated H_2SO_4. A chromotropic acid solution in concentrated H_2SO_4, 2 mg. per ml., is stable for at least three weeks. The intensity of the color produced by this solution increases with increments of 2,4-D according to Beer's Law (fig. 2).

[1] Paper no. 2817 of the Scientific Journal Series of the Minnesota Agricultural Experiment Station.

Chromotropic acid has been widely used as an analytical reagent in organic and biological chemistry following EEGRIWE's discovery (**6**) that it reacts with formaldehyde to give a red to purple color when heated in 72% H_2SO_4 solution. Among the aldehydes, the test was specific for formaldehyde and negative results were obtained with a variety of organic acids, ketones, sugars, and alcohols.

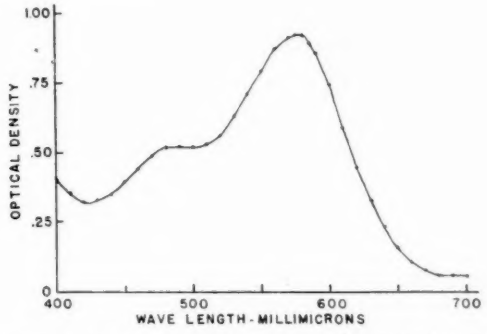

FIG. 1. Absorption spectrum of 2,4-D when reacted with chromotropic acid in concentrated sulphuric acid at 150 to 155° C for two minutes.

The reaction between chromotropic acid and formaldehyde has been applied to the qualitative and quantitative estimation of compounds which can be hydrolyzed, reduced or oxidized to yield formaldehyde. Formic acid (**6**), methanol (**1**), serine (**2**), diacetyl (**13**), methylal, pentaerythritol diformal, pentaerythritol monoformal, piperonal, n-propyl formal, and trioxane (**3**) have been determined qualitatively and/or quantitatively by means of this reaction. MACFAYDEN (**10**) and BOOS (**1**) have made quantitative determinations of formaldehyde with this reaction by reading extinction values at 580 mμ. BRICKER and VAIL (**5**) showed that chromotropic acid reacts with or retains the formaldehyde when a solution is evaporated to dryness and certain interfering organic compounds were removed. A num-

TABLE I

OPTICAL DENSITY RESULTING FROM THE REACTION OF VARIOUS CONCENTRATIONS OF CHROMOTROPIC ACID WITH 35 γ OF 2,4-D IN 2 ML. OF H_2SO_4.

Chromotropic acid	Optical density
mg.	
0.5	0.88
1.0	1.08
2.0	1.18
3.0	1.25
4.0	1.27
5.0	1.27

ber of organic compounds have been found to interfere with the reaction (**3, 4**). FREED (**7**) listed a number of other compounds which gave a color reaction with chromotropic acid.

The coincidence of the absorption maxima for the formaldehyde and 2,4-D color reactions as well as similarities in conditions under which the reactions are carried out suggests that the end products may be identical in each case, with formaldehyde ultimately reacting with the chromotropic acid. To obtain some information on the nature of the group or groupings involved in the 2,4-D reaction, qualitative tests were made with a number of compounds.

For preliminary tests, a few crystals of solid or 0.05 ml. of liquid compounds were treated with the reagents used for the quantitative determination of 2,4-D. Compounds which gave wine-purple colors were then ex-

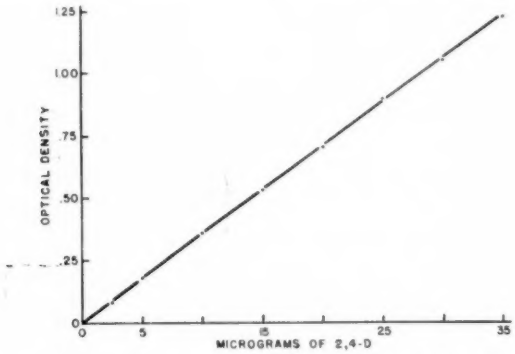

FIG. 2. Optical density of various concentrations of 2,4-D when reacted with 4 mg. of chromotropic acid in 2 ml. of concentrated sulphuric acid at 150 to 155° C for two minutes.

amined in a suitable concentration and absorption spectra were recorded of 11 of these compounds. Those that gave wine-purple colors were 2,4-D, phenoxyacetic acid, *o*-chlorophenoxyacetic acid, sodium 2,4-dichlorophenoxyacetate · H_2O, isopropyl ester of 2,4-D, monoethylamine salt of 2,4-D, monoisopropylamine salt of 2,4-D, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), β-naphthoxyacetic acid, methoxyacetic acid, glycolic acid, formaldehyde, trioxane, paraformaldehyde, methylal, piperonal, diacetyl, indoleacetic acid, and skatole. The absorption spectra of the solutions produced by these compounds were qualitatively similar to the spectrum of 2,4-D. All had absorption maxima at 490 and 580 mμ.

Compounds tested that did not produce a wine-purple color were phenylacetic acid, α-phenoxyproprionic acid, phenoxyacetamide, anisole, benzoic acid, *o*-chlorobenzoic acid, phenol, toluene, trans cinnamic acid, 2,4-dichlorophenol, α-naphthaleneacetic acid, α-naphthaleneacetamide, formic acid, ox-

alic acid, citric acid, monochloroacetic acid, acetic acid, methyl alcohol, succinic acid, glycine, diethyl ether, indole, tryptophan, β-3-indolepropionic acid, and γ-3-indolebutyric acid.

Although it may be suggested, on the basis of the similarity of the absorption spectra, that formaldehyde is responsible for the color produced in all cases, definite proof of this fact has not been established. If it is assumed that one molecule of formaldehyde is formed for every molecule of 2,4-D, the molecular extinction coefficients of the colored compound obtained from formaldehyde or from 2,4-D should be the same and should hold for the other compounds which react to yield one molecule of formaldehyde. Molecular extinction coefficients based on an average of two to four determinations were calculated. The length of the light path was 1.0 cm. Since the exact concentration of the available formaldehyde was not

TABLE II

MOLECULAR EXTINCTION COEFFICIENTS AT 580 MILLIMICRONS OF COMPOUNDS WHICH PRODUCED WINE-PURPLE COLORS WITH CHROMOTROPIC ACID IN CONCENTRATED SULPHURIC ACID.

Compounds	Concentration	Optical density	Molecular extinction coefficient
	mg./ml.		
Formaldehyde	.00175	.97	16,600
2,4-dichlorophenoxyacetic acid (2,4-D)	.0125	.92	16,300
Glycolic acid	.0038	.80	16,000
Sodium 2,4-dichlorophenoxyacetate $\cdot H_2O$	.0125	.77	16,100
Monoethylamine salt of 2,4-D	.0125	.75	16,000
Monoisopropylamine salt of 2,4-D	.0125	.71	15,900
2,4,5-Trichlorophenoxyacetic acid	.0125	.79	16,100
Isopropyl ester of 2,4-D	.0125	.57	12,000
Phenoxyacetic acid	.0125	.95	11,500
o-Chlorophenoxyacetic acid	.0125	.76	11,300
Indoleacetic acid	.0125	.66	9,300
β-Naphthoxyacetic acid	.0125	.45	7,300

known, the calculation of its extinction coefficient was based on its polymer trioxane which yields 3 molecules of formaldehyde upon hydrolysis.

The results of the calculations are summarized in table II. The values obtained are not absolute since they were determined with round rather than square cuvettes. The relative values are valid for purposes of comparison, however, and close agreement is found in the case of seven of the substances examined. The values for the other compounds are lower than theoretically expected if the assumption is correct that one mole of formaldehyde is produced for one mole of substance. The lower values could be due to impurities, interfering substances, side reactions or other causes.

Chromotropic acid has been used extensively in qualitative and quantitative inorganic analysis. Reactions of chromotropic acid are known with titanium salts, chromic acid, chromates, dichromates, mercuric salts, ferric

salts, uranyl salts, neutral tungsten, silver, nitrate and nitrite ions (**9, 14, 15**). Since chromotropic acid reacts with many inorganic ions in aqueous solution, the reactivity of various ions in concentrated sulphuric acid was also investigated. Nitrate, nitrite, chromate, and dichromate reacted to give colors in the visible range under the conditions of the test. The following ions did not react to give colored reaction products with chromotropic acid in sulphuric acid: Fe^{++}, Fe^{+++}, Cl^{-}, Hg^{++}, $SO_4^{=}$, $PO_4^{\equiv}$, K^{+}, Mg^{++}, Ca^{++}, Na^{+}, NH_4^{+}, Ag^{+}, UO_2^{++}. It was not determined, however, if these ions interfere with color formation.

Since the completion of this work, SLEPECKY and BECK (**12**) have referred in an abstract to their procedure for a quantitative adaptation of Freed's method of detection of 2,4-D. MARQUARDT and LUCE (**11**) published the details of their procedure for determination of 2,4-D in milk. While the procedure reported here has not been used for the determination of 2,4-D in materials of plant or animal origin, it appears to have the potential advantages of greater sensitivity and greater stability of reagents when compared with the method of Marquardt and Luce.

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EXTRACELLULAR PHOTOSYNTHETIC REACTIONS. II. IDENTIFICATION OF RADIOACTIVE MALATE

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(WITH ONE FIGURE)

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It was independently shown in three laboratories (**14, 11, 2**) that illuminated, isolated chloroplasts can reduce triphosphopyridine nucleotide (TPN) and bring about a reductive carboxylation of pyruvate with the aid of malic enzyme derived from several sources: animal (**14**), non-photosynthetic plant tissue (**11**) and green leaves (**2**). The overall reaction was

$$\text{Pyruvate} + CO_2 + H_2O \rightarrow \text{malate} + 1/2\ O_2.$$

The photochemical reduction of TPN, and also of diphosphopyridine nucleotide (**13**), permits the linking of the photolysis of water by isolated chloroplasts (the Hill reaction) to the many enzymatic reactions which are dependent on these pyridine nucleotides (**10**). Among these reactions, those which can be shown to occur in photosynthetic tissues are of special interest to photosynthesis.

In the previously reported study (**2**) in which chloroplasts and malic enzyme from green leaves were used, the course of the reaction was followed by manometric measurements of the evolved oxygen. This communication reports the identification, with the aid of radioactive carbon, paper chromatography, and radioautography, of the organic compound, l-malate, formed in this photosynthetic reaction. Tracer carbon was previously used in the study of the reaction with malic enzymes from non-photosynthetic tissues (**14, 11**), and the identification of l-malate as a product of the reaction with the pigeon liver enzyme was reported by VISHNIAC and OCHOA (**14**), who used both chromotographic techniques and specific enzymatic decarboxylation methods.

Previously described procedures (**1, 4**) were used for the growing of sugar beet and spinach plants in nutrient solutions in a greenhouse and for the maceration of leaves and the separation of chloroplast fragments from the cytoplasmic fluid. In the early experiments, malic enzyme was prepared from the cytoplasmic fluid in which the activity of the enzyme was found to be localized (**2**). In later experiments, the enzyme was prepared from the green slurry of the entire leaf; the chloroplastic material was removed with the first ammonium sulphate precipitation. The malic enzyme and the chloroplasts were usually prepared from the same leaves, but in certain experiments spinach chloroplasts were combined with malic enzyme of sugar beet. The procedure used for the preparation of malic enzyme was based on that of VENNESLAND (**13**) for the preparation of oxalacetic car-

boxylase with which malic enzyme activity is associated. Large, healthy leaves were placed in a water-proof plastic bag, chilled for two hours at around 5° C and minced (**4**). The slurry was filtered through cotton and cheesecloth, centrifuged for one minute at 2000 r.p.m. and a radius of 14 cm. and the residue discarded. The supernatant fluid was brought to 33% saturation with solid ammonium sulphate, allowed to stand for 15 to 20 minutes and was centrifuged as above for 25 to 30 minutes. The residue was discarded, and the supernatant fluid was brought to 66% saturation with solid ammonium sulphate, allowed to stand for one hour, and then centrifuged as above for 30 minutes. The supernatant fluid was discarded and the residue which contained the enzyme was dissolved in a small volume of 0.025 M phosphate buffer, pH 7.4, and dialyzed against the same buffer until free from ammonium sulphate. All operations were carried out in the cold.

The experiments were carried out in conical manometer vessels of about 16 ml. capacity. In the main compartment of the vessel were placed washed chloroplast fragments containing 0.5 mg. chlorophyll, 20 micromoles potassium chloride, 10 micromoles manganous chloride, 100 micromoles lithium pyruvate, 30 micromoles sodium bicarbonate, 0.3 micromole triphosphopyridine nucleotide (TPN), 100 micromoles tris hydroxymethylaminomethane buffer of pH 7.5 and water to give a final volume of 3 ml. The side arm contained a radioactive sodium bicarbonate solution of 7.4 micromoles of CO_2 with a total count of around 700,000 counts per minute. The gas phase in the vessels was 5% CO_2 and 95% N_2, the temperature was 20° C, and the pH was 7.4. In the treatment in which light was excluded the vessel was wrapped in tinfoil. The preparation of washed chloroplast fragments, the light intensity and other experimental conditions were as previously described (**2, 4**).

The details of technique varied somewhat in different experiments. In the representative experiment from which the data presented here were taken, the following procedure was followed. After flushing the vessels with the CO_2-N_2 gas mixture and temperature equilibration, the radioactive bicarbonate solution was added from the side arm to the reaction mixture. An additional period of equilibration in the dark was allowed, following which the vessels were exposed to light for 12 minutes with continuous shaking. Oxygen was measured manometrically during the course of the reaction (**2**). At the end of the exposure to light, the vessels were detached from the manometers and the reaction mixture was transferred to small glass centrifuge tubes and acidified with H_2SO_4 to pH 3. The acidified mixture was heated with shaking in a boiling water bath for five minutes, cooled and filtered. The clear filtrate which contained all of the radioactivity was reduced in volume to 0.25 ml. under a heat lamp and aliquots of 0.03 to 0.04 ml. were used for counting radioactivity or for paper chromatography. In the former case, the aliquots were thinly spread on metal disks, dried and counted directly using a thin window (1.4 mg./cm.^{2}) tube.

For paper chromatography, successive 0.01-ml. portions were placed on Whatman no. 1 filter paper sheets and dried with a fan.

One-dimensional paper chromatography with butanol-formate (**9**) solvent was used for the identification of malic acid. The solvent was prepared by mixing in a large separatory funnel equal volumes of 4 M formic acid and normal butanol. The mixture was allowed to stand with occasional shaking for several days prior to the removal of the butanol layer for use. The filter paper sheets were suspended in metal cabinets with the aid of glass rods from Pyrex glass troughs containing the solvent which flowed downward through the paper.

Several aliquots of the reaction mixture were conveniently spaced on the same filter paper sheet. To some of them were added 40 micrograms of authentic l-malic acid for the purpose of co-chromatography. Included on the same paper were aliquots of l-malic acid applied singly or mixed with several other authentic dicarboxylic acids. The position of malic acid on the chromatogram was established by color spots obtained from authentic malic acid after spraying the dry paper with bromphenol blue indicator

TABLE I

PHOTOCHEMICAL $C^{14}O_2$ FIXATION AND O_2 EVOLUTION BY A MIXTURE OF CHLOROPLAST FRAGMENTS AND LEAF MALIC ENZYME.

Treatment	Net count of aliquot as per cent. of background	Total $C^{14}O_2$ fixed	Oxygen evolved
		counts/minute	*mm.*^{3}
Complete system in light	970	2,615	18
TPN omitted	33	122	....
Malic enzyme omitted	80	290	15
Complete system in dark	....	....	....

(**9**). The amount of photosynthesized malate was small; but it was readily identified by the technique of radioautography (**3**), which was first applied to paper chromatography by FINK *et al.* (**8**) and used extensively by BENSON *et al.* (**7**) for the identification of photosynthetic products. The position of the radioactive malate spot as shown on the x-ray film coincided with the position of the color spot of authentic malate on the filter paper (fig. 1).

Table I shows that a significant fixation of carbon dioxide occurred only in the complete reaction mixture in the light. The second column of table I was computed by subtracting the background count from the total count of the respective aliquots and expressing the difference as a percentage of the background count. The significance of the net count of the aliquot from the complete, illuminated treatment is beyond question; the count was about 10 times that of the background. The net count, however, when either TPN or malic enzyme was omitted, is not considered significant. In both cases the actual net count of the aliquot was less than that of background. As shown in figure 1, a radioactive malate spot was obtained in the complete treatment after an exposure of 18 days. No radiocarbon fix-

ation could be detected when either TPN or malic enzyme were omitted by the radioautographic technique even after an exposure of 52 days. The complete system fixed CO_2 only when exposed to light; no fixation of radiocarbon could be detected in the dark.

With regard to oxygen, table I indicates that the highest production of oxygen was obtained from the complete illuminated system. However, when malic enzyme was omitted there was only a slight reduction in oxy-

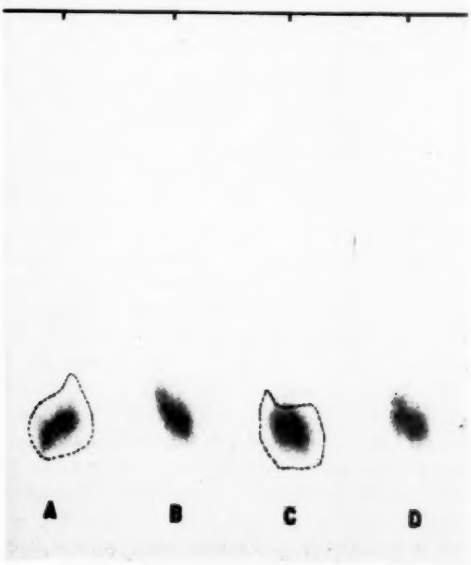

FIG. 1. Radioautograph of photosynthesized malic acid separated by means of one-directional paper chromatography. Replicate aliquots A, B, C, D of the reaction mixture were placed on filter paper at line of origin. A and C were combined with 40 micrograms of authentic l-malic acid. Dark spots A, B, C, and D are radioautographs of photosynthesized malic acid. Dotted lines at A and C represent the boundaries of the coincident color spots obtained by spraying with indicator the photosynthesized malic acid which was co-chromatographed with the authentic compound.

gen evolution, despite the lack of malate synthesis. This result indicates that under the conditions of the experiment TPN acted as a hydrogen acceptor in the photolytic cleavage of water. On the basis of molar concentrations present, about five times as much oxygen was evolved as would be expected from the TPN present which suggests that the reduced TPN was cyclically oxidized by some oxidant present in the reaction mixture. TOLMACH (**12**), using a sensitive technique for the measurement of very small amounts of evolved oxygen, observed an enhanced evolution of oxygen by isolated chloroplasts upon adding TPN without the malic enzyme system.

Additions of small amounts of TPN resulted under his conditions in yields of oxygen 30 to 40 times greater than the stoichiometric amounts of TPN added.

The amounts of malate fixed were small but significant. A computation based on the amounts of radiocarbon fixed indicates a total fixation of CO_2 of 0.2 micromole which would correspond to 4.5 mm.^{3} of oxygen. Table I shows a net evolution of 3 mm.^{3} of oxygen in the complete reaction mixture which gave malate synthesis. This figure for oxygen is obtained by subtracting from the total oxygen evolution the very large blank given by the system without malic enzyme in which TPN was present but no malate synthesis took place. Thus, the relative amounts of CO_2 fixed and oxygen evolved were approximately what could be expected; but since they were small, it is doubtful whether great significance can be attached to this comparison.

The main objective of the investigation as reported in this and a previous paper (**2**) was the reconstruction from components of green leaves of a system capable of capturing and transforming light into chemical energy for the reduction of CO_2 with a simultaneous evolution of oxygen. The malic enzyme preparation and the chloroplast fragments were obtained directly from leaves; the other constituents of the reaction mixture are known to occur in leaves (**2**). The Hill reaction shows that isolated chloroplasts can utilize the energy of light for the photolysis of water with a resultant evolution of gaseous oxygen, but the capacity to use light energy for the reductive fixation of CO_2 remained a property of the intact green cell. The data in Table I and figure 1 indicate that the photosynthetic apparatus of green leaves can be taken apart and put together outside the cell to give a photochemical reaction characterized by the same two simultaneous events which characterize photosynthesis in the intact cell: fixation of CO_2 and evolution of oxygen.

The question arises whether malic acid is one of the main pathways of photosynthesis *in vivo*. Badin and Calvin (**5**) presented kinetic evidence in favor of two main pathways of carbon dioxide fixation in photosynthesis: one, through malate, operative under conditions of low light intensity and another, through phosphoglycerate, operative under conditions of high light intensity. In another paper from the same laboratory (**6**) Bassham *et al.* concluded from malonate inhibition studies that malate is not the precursor of the α- and β-atoms of phosphoglyceric acid. The elucidation of the precise role of malate in the overall scheme of photosynthesis must await further experimentation.

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DIORTHO SUBSTITUTED PHENOXYACETIC ACIDS AS ANTIAUXINS

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(WITH ONE FIGURE)

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This paper concerns a new class of antiauxins, substances in themselves devoid of activity in the Avena coleoptile section growth test but capable of antagonizing the growth promoting effects of both native and synthetic auxins. These compounds, to which antiauxin activity has not previously been ascribed, are the diortho substituted phenoxyacetic acids. To establish rigorously that a growth inhibiting agent acts by antagonizing the action of auxins, rather than in some other manner, it is necessary to apply a kinetic analysis such as that of Lineweaver and Burk (**2**). This treatment was originally designed to apply to the study of isolated enzyme systems. It will be shown in the present paper, however, that the growth evoked by the application of auxin to a particular plant tissue, the Avena coleoptile section, can be described and treated by the methods of enzyme kinetics. With the aid of this treatment it will be shown that the diortho substituted phenoxyacetic acids competitively and reversibly inhibit the action of indoleacetic acid (IAA) and of 2,4-dichlorophenoxyacetic acid (2,4-D).

Avena seedlings (var. Siegeshafer) were grown in vermiculite contained in stainless steel flats and were incubated under red light (with occasional orange light) at 25 to 26° C for 82 to 84 hours. At this time those plants with coleoptiles 2.75 to 3.25 cm. in length were selected for use. One section 5.0 mm. in length, taken 2 to 3 mm. from the tip, was cut with a double bladed cutting tool from each hollow coleoptile cylinder. Each treatment was carried out with 20 such sections, removed after randomization of the entire lot, and floated in Petri dishes containing 20 ml. of solution. The basic medium consisted of Pyrex redistilled water, 3% sucrose and 0.0025 M potassium maleate buffer, pH 4.5. The sections were incubated in the dark at 25 to 26° C. Growth under these conditions is linear with time for approximately 18 to 24 hours. The measurements recorded in this paper were made after incubation for 12 hours and are, therefore, measurements of initial growth velocity. The standard error for the growth of 20 sections within one treatment was on the average 2.1% of the mean. Duplicate means based on 20 sections differed from one another by an average of 3.1%.

The influence of varying concentrations of IAA and of 2,6-dichlorophenoxyacetic acid (2,6-DCPA) alone and in combination, on the growth of Avena coleoptile sections is shown in table I. It is clear that 2,6-DCPA is itself inactive over the concentration range tested. It is also apparent that 2,6-DCPA inhibits the section growth induced by IAA and that this inhibition increases with increasing concentrations of 2,6-DCPA. The inhibition

by any given concentration of 2,6-DCPA does, however, decrease as the concentration of IAA is increased.

That 2,6-DCPA competitively inhibits the action of IAA is shown by application of the Lineweaver-Burk formulation for competitive systems. For this treatment which is shown in figure 1 A, the reciprocal of the growth rate is plotted as a reciprocal of the concentration of IAA. The lower line is that obtained from the data on growth with IAA alone while the upper lines are those obtained from the data on growth in the presence of IAA but with increasing concentrations of 2,6-DCPA. The intercept of each line on the ordinate is a measure (1/growth) of the maximum growth rate of which the system is capable. That the lines have a common intercept shows that the growth inhibition induced by varying concentrations of the antiauxin can be overcome provided that sufficient IAA is applied. The fact that the data of table I are so precisely compatible with the Lineweaver-Burk for-

TABLE I

EFFECT OF 2,6-DCPA ON GROWTH OF AVENA COLEOPTILE SECTIONS INDUCED BY IAA.

Concentration of 2,6-DCPA	Concentration of IAA					
	mg./l.					
	0.00	0.02	0.05	0.1	0.5	1.0
	Growth in absence of IAA	Growth in presence of IAA minus growth in absence of IAA				
mg./l.	*mm./section/12 hrs.*	*mm./section/12 hrs.*				
0.0	0.42	0.66	1.17	1.52	2.17	2.50
0.1	0.42	0.56	1.00	1.39	2.10	2.48
0.5	0.43	0.46	0.88	1.25	2.01	2.43
1.0	0.41	0.36	0.71	1.12	1.92	2.38

mulation suggests then that the inhibition of 2,6-DCPA is in fact a competitive one and that 2,6-DCPA is, therefore, a true antiauxin.

Entirely similar results for the inhibition of 2,6-DCPA of section growth induced by 2,4-D are shown in table II. The Lineweaver-Burk treatment of the data of table II is given in figure 1 B and shows that this inhibition is also a competitive one. Therefore, 2,6-DCPA is an antiauxin toward 2,4-D as well as toward IAA.

Trichlorophenoxyacetic acid (2,4,6-TCPA), like its analog 2,6-DCPA, is an inhibitor of section growth induced by auxin. The Lineweaver-Burk treatment for the data on the interaction of 2,4,6-TCPA with IAA and 2,4-D are presented in figure 1 C and 1 D, respectively. It is evident that, again, the criteria for competitive inhibition are fulfilled with precision.

The interrelationships of auxins and antiauxins in Avena section growth in the quantitative terms which are used for the description of such relations in classical enzyme kinetics will now be considered. Two parameters are required to characterize the activity of a substrate, in this case auxin, toward an enzyme system, in this case the coleoptile section. These parame-

ters are, respectively, the K_s or dissociation constant of the enzyme-substrate complex and the V_{max} or maximum velocity with which the enzyme can attack and alter the substrate. The K_s may be defined in the present instance as that concentration of auxin required to half-saturate the growth-system enzyme and is a measure of the affinity of the enzyme for its substrate. The V_{max} or maximum reaction velocity represents the rate at which the reaction proceeds when all the enzyme molecules are saturated with substrate and is in this case the maximum initial growth velocity in the

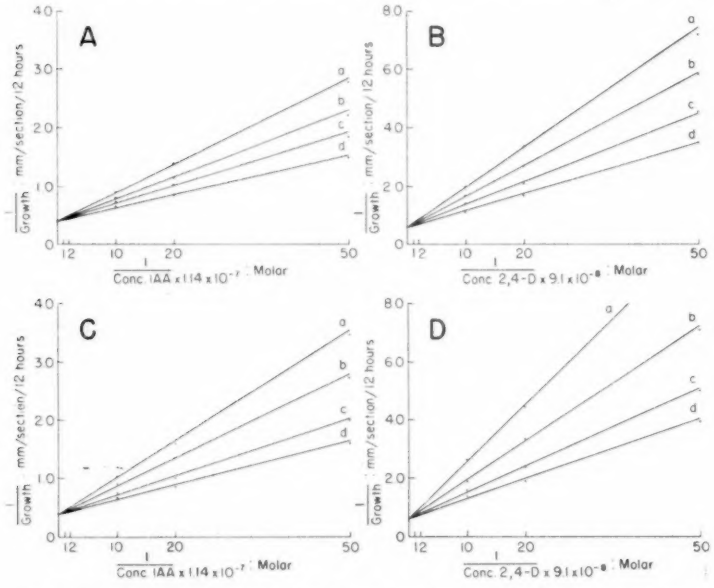

FIG. 1. Inhibition of auxin induced Avena section growth by diortho substituted antiauxins. Data plotted according to the Lineweaver-Burk treatment as a test of competitive inhibition. A and B. 2,6-DCPA as inhibitor. C and D. 2,4,6-TCPA as inhibitor. A and C. IAA as auxin. B and D. 2,4-D as auxin. In A and B, curves a, b, c and d refer to inhibitor concentrations of 4.52, 2.26, 0.452 and 0.00×10^{-6} M, respectively. In C and D, curves a, b, c, and d refer to inhibitor concentrations of 3.92, 1.96, 0.392 and 0.00×10^{-6} M, respectively.

presence of non-limiting auxin concentrations. Just as the K_s characterizes the affinity of the substrate (auxin) for its enzyme (section) so the K_I or inhibition constant characterizes the affinity of a competitive inhibitor for this same enzyme. Since the K_I is a property of inhibitor and enzyme alone, for K_I does not depend on the nature or even on the presence or absence of the substrate, it follows that if the coleoptile section auxin—antiauxin system is a truly competitive one, K_I for a given inhibitor should be independent of the nature of the auxin present. Thus the K_I of 2,6-DCPA, for

TABLE II

EFFECT OF 2,6-DCPA ON GROWTH OF AVENA COLEOPTILE SECTIONS INDUCED BY 2,4-D.

Concentration of 2,6-DCPA	Concentration of 2,4-D					
	mg./l.					
	0.00	0.02	0.05	0.1	0.5	1.0
	Growth in absence of 2,4-D	Growth in presence of 2,4-D minus growth in absence of 2,4-D				
mg./l.	*mm./section/12 hrs.*	*mm./section/12 hrs.*				
0.0	0.42	0.29	0.59	0.78	1.39	1.58
0.1	0.42	0.22	0.47	0.71	1.34	1.58
0.5	0.43	0.17	0.37	0.60	1.27	1.52
1.0	0.42	0.14	0.30	0.50	1.18	1.48

example, should be the same whether the compound is used to inhibit growth induced with IAA or growth induced with 2,4-D. That the value of K_I is the same is shown by the data of table III. This table gives, first, the experimental values for K_s and V_{max} of IAA and of 2,4-D obtained from the data of figure 1. It can be seen that the K_s for IAA is slightly lower than that for 2,4-D. The V_{max} for IAA is also considerably greater than that for 2,4-D. The second portion of the table shows that the K_I for 2,6-DCPA or 2,4,6-TCPA in the presence of IAA is substantially identical with that which obtains in the presence of 2,4-D. The K_I of 2,4,6-TCPA is approximately one half that of 2,6-DCPA and 2,4,6-TCPA is, therefore, a more effective antiauxin than is 2,6-DCPA.

It is evident that there are intimate relationships between those molecules which possess auxin activity and closely related molecules which are antiauxins. Thus, 2,4-D may be converted into an antiauxin either by the introduction of a substituent at position 6 as in 2,4,6-TCPA or by removal of the carboxyl group as in 2,4-dichloranisole (**1**). These relationships may be understood in the light of the two-point attachment concept of MUIR *et al.* (**3**) according to which an auxin molecule undergoes attachment at the appropriate site within the plant, not only through the carboxyl group but

TABLE III

CONSTANTS WHICH DESCRIBE THE ACTIVITIES OF CERTAIN AUXINS AND ANTIAUXINS IN TERMS OF ENZYME KINETICS.

	K_s (molar)	K_I (molar)	V_{max}
			mm./section/12 hrs.
IAA	3.55×10^{-7}		2.60
2,4-D	5.15×10^{-7}		1.76
2,6-DCPA			
in presence of IAA		4.0×10^{-6}	0.00
in presence of 2,4-D		3.7×10^{-6}	0.00
2,4,6-TCPA			
in presence of IAA		2.0×10^{-6}	0.00
in presence of 2,4-D		2.1×10^{-6}	0.00

also through substitution at the reactive ortho position. Removal of either of these essential groups not only abolishes the auxin activity of 2,4-D but also converts the molecule into an effective antiauxin. It would appear probable, according to the kinetic analysis given above, that in these instances the antiauxin molecule exerts its effect by one-point attachment at the respective site thus blocking the consummation of two-point attachment by active auxin molecules. It is not only possible but also probable that there are still other structural modifications which may confer antiauxin activity upon an auxin molecule. Among such modifications may be the disturbance of the spatial relationship between the carboxyl group and the ortho position as has been reported by VAN OVERBEEK *et al.* (**4**) for the trans isomers of compounds active in the cis form. In this case also, antiauxin activity may be due to the inability of the auxin analog to consummate two-point attachment, an inability caused by the spatial arrangements of the trans configurations.

The work presented in this report was supported in part by the Herman Frasch Foundation.

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NOTES

A.S.P.P. Election.—The results of the annual election of the American Society of Plant Physiologists for 1952–53 are as follows: President, Dr. Daniel I. Arnon, University of California, Berkeley, California; Vice-President, Dr. Folke Skoog, University of Wisconsin, Madison, Wisconsin; Members of the Editorial Board, Dr. James Bonner, California Institute of Technology, Pasadena, California; and Dr. Walter E. Loomis, Iowa State College, Ames, Iowa.

The New England Section.—The New England Section of the A.S.P.P. held its annual gathering on the campus of the University of Maine, May 16–17. Dr. F. H. Steinmetz and his coworkers planned the program in which representatives from Canadian and European universities also participated. The officers elected by the New England Section for 1952–53 are Chairman, C. J. Lyon, Dartmouth College; Vice-Chairman, K. E. Wright, Smith College; Secretary-Treasurer, L. H. Jones, University of Massachusetts.

Warren B. Mack.—Dr. Warren B. Mack, Professor of Horticulture and Head of the Department of Horticulture at The Pennsylvania State College, a distinguished horticulturist and plant physiologist, died in a Philadelphia hospital on July 6, 1952. Death was caused by a coronary occlusion. For nearly 30 years Professor Mack's life and work has exerted a profound and lasting influence on horticultural practices and production.

Warren Mack spent the early years of his life on the family farm at Flicksville, Pennsylvania, where he received his primary education before enrolling at Lafayette College from which he graduated with first honors and election to Phi Beta Kappa in 1915. After three years of teaching science at the Manasquan, New Jersey, high schools, followed by service as 2nd lieutenant in the field artillery of the U. S. Army during World War I, he decided to embark on a career in horticulture, for which purpose he entered The Pennsylvania State College in 1919, graduating with the B.S. degree in Horticulture in 1921.

After a period (1921–23) of teaching pomology at the Massachusetts Agricultural College (now the University of Massachusetts), he returned to The Pennsylvania State College as instructor in horticulture, advancing in seven years to a professorship in the division of vegetable gardening, and appointment as Head of the Department of Horticulture in 1937.

Professor Mack interrupted his service at the College in 1927 to take graduate study at Johns Hopkins University where he obtained his doctorate in 1929 under the guidance of the distinguished plant physiologist, Burton E. Livingston. Livingston had considerable influence in the formation of Mack's ideas on the relation of fundamental research to agricultural practice. Here began the fruitful series of investigations, continued on his

return to the College, on the relation of oxygen pressure to the influence of ethylene on CO_2 production by plants—experiments which revealed the cause of the then prevailing discrepancies and disagreements on ethylene effects in plant and animal tissues.

Up to the time of his appointment as Head of the Department in 1937, notwithstanding a heavy teaching schedule, Warren Mack's inquiring mind kept him active in numerous research projects. These included investigations on the bearing habits of apple varieties; quantitative studies of form and size in vegetables; and a field project on the influence of fertilizers on the moisture content of soils. Another investigation carried out during this period on the effects of nitrogen fertilization on greenhouse tomatoes indicated—contrary to the then widely accepted prevailing view—that nitrogen fertilization in amounts ranging from medium to very heavy, or in repeated application did not induce an over-vegetative condition in greenhouse tomato plants grown in composted soils in ground beds, and showed the danger of broad generalization in the complicated field of plant soil interrelationship. Other investigations during this period included a project on the influence of potassium iodide on the iodine content of beans, turnips and other vegetables, and also one on the effect of potassium salts on the availability of nitrogen on ammonium nitrate.

Difficulties met with in interpreting some of the results of these field experiments, particularly those of a long-continued experiment on the fertilization of truck crops in rotation, forcibly brought to Mack's attention the realization of the limitations of *ad hoc* solutions in field experiments through the measurement of yields alone on a multiplicity of fertilizer field plots. He felt the need for a search of principles and their application to such problems. Thus began the intimate association from 1937 until his death between Warren and the writer in a series of investigations on the application of plant leaf composition to determine the nutrient requirements of plants and the relation between nutrition and the growth and development of crops. His last paper, for example, which was given at a symposium at the Philadelphia meeting was on plant composition as an index of the nutrient requirements of horticultural crops.

Professor Mack was an excellent teacher and also inspired confidence and good will in his students. In addition to his heavy administrative duties, he found time to advise students with their problems and also to teach an undergraduate and graduate course in horticulture.

It is natural that his accomplishments and restless activity would gain him wide recognition and many honors, among which may be mentioned the award of the Honorary Doctor of Science by Lafayette College in 1946, President of the American Society for Horticultural Science in 1944–46, Executive Secretary State Victory Garden Commission Pennsylvania State Council of Defense 1942–45, election to Phi Kappa Phi associates in 1950, and to the National Academy of Design. The latter honor was a tribute to his international recognition as an expert craftsman in the graphic arts in which he was interested since boyhood. Since 1931 his wood engravings

have been exhibited in many of the leading galleries including the Metropolitan Museum, Carnegie Institute, Smithsonian Institute of Washington and the Library of Congress. Many of his prints have received high honors including the Manning Purchase Prize (1938), Appalachian Museum Purchase Prize (1940) and many others.

Warren Mack was married in 1923 to Pauline Beery, a member of the faculty of the College from 1919 until she resigned in June 1952, as professor of household chemistry and director of the Ellen Richards Institute, to accept the deanship of the newly established College of Household Arts and Sciences of the Texas State College for Women at Denton, Texas. Unwilling for separation from his wife, Dr. Mack also requested retirement from the College, and this was approved effective August 31, 1952. Warren had planned to devote his time to writing and his art work at Denton, and had last November asked the writer to cooperate with him in a projected book on the nutrition of horticultural crops.

Besides his wife, his mother, a brother and a sister survive him. The influence of Warren Mack's life has not come to a close with his death; his spirit will live in the minds and hearts of all his friends.—WALTER THOMAS.

Annual Review of Plant Physiology, Volume III.—D. I. ARNON, Editor. Annual Reviews, Inc., Stanford, California. 369 pages, 1952. $6.00.

The reviews appearing in the third volume of this series are as follows: Factors Affecting Availability of Inorganic Nutrients in Soils with Special Reference to Micro-Nutrient Metals by G. W. Leeper; Glycolytic Enzymes in Higher Plants by P. K. Stumpf; Metabolism of Phosphorylated Compounds in Plants by H. Albaum; Studies of the Physiology, Pharmacology, and Biochemistry of the Auxins by J. Bonner and R. S. Bandurski; Agricultural Application of Growth Regulators and Their Physiological Basis by J. van Overbeek; Transport of Organic Compounds by W. H. Arisz; Leaf Proteins by S. Wildman and A. Jagendorf; Physiological Aspects of Low Temperature Preservation of Plant Products by M. A. Joslyn and H. C. Diehl; Physiology of Virus Diseases by F. C. Bawden and N. W. Pirie; Mechanisms of Ion Absorption by Roots by R. Overstreet and L. Jacobson; Physiological Bases for Assessing the Nutritional Requirements of Plants by A. Ulrich; Photosynthesis by E. Rabinowitch; Physiology of Flowering by A. Lang; Carboxylating Enzymes in Plants by B. Vennesland and E. E. Conn; Tree Physiology by B. Huber. Author and subject indices are included.

Advances in Enzymology and Related Subjects of Biochemistry, Volume XIII.—Edited by F. F. NORD. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, New York. 413 pages, 1952. $8.50.

The thirteenth volume in this series of publications contains the following articles: Localization of Enzymes in Cytoplasm by H. Holter; Some Aspects of the Application of Tracers in Permeability Studies by H. H.

Ussing; La Biosynthèse Induite des Enzymes (Adaptation Enzymatique) by J. Monod and M. Cohn; Genetic Formulation of Gene Structure and Gene Action by G. Pontecorvo; Adenosine Triphosphate and the Structural Proteins in Relation to Muscle Contraction by D. M. Needham; Hyaluronidases by K. Meyer and M. M. Rapport; Certain Aspects of the Intermediary Metabolism of Glutamine, Asparagine, and Glutathione by H. Waelsch; Stoichiometric Inhibition of Chymotrypsin by A. K. Balls and E. F. Jansen; The Comparative Biochemistry of Nitrogen Fixation by P. W. Wilson. Author and subject indices for this volume and a cumulative index of authors and subject for volumes one to thirteen complete the book.

Soil Physical Conditions and Plant Growth.—B. T. Shaw, Editor. Academic Press, Inc., 125 East 23rd Street, New York 10, New York. 491 pages, 1952. $8.80.

This monograph is the second in a series prepared under the auspices of the American Society of Agronomy. Its purpose is to evaluate the relation of physical conditions of the soil to plant growth. The monograph will be particularly helpful to advanced students in soil physics and plant physiology. The chapters of the monograph are Soil as a Physical System by L. T. Alexander and H. E. Middleton; Mechanical Impedance and Plant Growth by J. F. Lutz; Soil Water and Plant Growth by L. A. Richards and C. H. Wadleigh; Soil Aeration and Plant Growth by M. B. Russell; Soil Temperature and Plant Growth by S. J. Richards, R. M. Hagan, and T. M. McCalla. An index completes the volume.

The Pectic Substances.—Z. I. Kertesz. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, New York. 628 pages, 1951. $13.50.

This comprehensive monograph is an attempt to bring together the information on the pectic substances which is widely scattered in the publications of various branches of science and technology. It has been written for those who are interested in problems related in any manner to the pectic substances rather than the specialists in pectin chemistry. The subject matter is arranged in five parts dealing with chemistry, botany, biochemistry and manufacture of pectic substances and some functions and applications of pectic substances and pectic enzymes.

The Action of Hormones in Plants and Invertebrates.—Edited by K. V. Thimann. Academic Press, Inc., 125 East 23rd Street, New York 10, New York. 228 pages, 1952. $5.80.

This book is a reprint of four chapters from a comprehensive treatise on hormones which appeared in 1948. Supplementary bibliographies listing papers appearing in the intervening years through 1950 have been added to each chapter. The chapters are Plant Growth Hormones and Other Plant Hormones by K. V. Thimann; Hormones in Insects by B. Scharrer; Hormones in Crustaceans by F. A. Brown, Jr.

Die Wuchsstofflehre. Ergebnisse und Probleme der Wuchstofforschung.—H. Söding. Georg Thieme Verlag, 47 Diemershaldenstrasse, Stuttgart, Germany. 304 pages, 1952. DM 33.

A treatise of this kind is valuable for its review of the literature available to the author, the integration it attempts, and the viewpoint it expresses. Of special interest to plant scientists in this country is the consideration given papers on growth regulators published in Europe in recent years and the divergent views that have developed. Professor Söding is of the opinion that indoleacetic acid acts only as an activator of auxins. The book discusses methods of investigation of growth regulators; the formation and distribution of growth hormones in the plant; the hormone content of normal and pathological plant tissues; translocation of growth regulators; the chemistry of auxins and antagonists; the role of auxin in orientation movements of plants, cambial activity, tumor formation, root formation, correlative inhibition including fruit and leaf fall, regeneration, bud development, and germination; the effect of auxins on the cell; the position of growth hormones in the entire system of plant hormones; and the interrelation of action of all hormones in plants. A subject index is included in the book.

Botany of Sugarcane.—C. van Dillewijn. Chronica Botanica Company, Waltham, Massachusetts. 371 pages. 1952. $6.00.

This volume gives detailed consideration to the morphology, anatomy, and physiology of the sugar-cane plant. Separate chapters of the section on physiology describe germination of seeds, propagation by cuttings, tillering, growth, vegetative composition, chemical composition, mineral nutrition, water relationships, photosynthesis and respiration. The bibliography lists 617 publications relating to sugar cane. A subject index completes the book.

Deformation and Flow in Biological Systems.—A. Frey-Wyssling, Editor. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, New York. 552 pages, 1952. $11.50.

This volume is one of a series of monographs on the rheological behavior of natural and synthetic products. Although phenomena of deformation and flow are evident in biological systems, fully quantitative rheological treatments are not possible at the present time. The 10 essays of this monograph describe various aspects of biological systems and outline the problems awaiting rheological analysis. The essays on rheology in cells are The Rheological Properties of Protoplasm by W. Seifriz; The Rheology of Muscle by M. G. M. Pryor; and Deformation of Plant Cell Walls by A. Frey-Wyssling. The articles on translocation of solutes in plants are Movement of Water in Higher Plants by R. D. Preston; Latex Flow by A. Frey-Wyssling; and Viscous Flow through Elastic Capillaries by J. J. Hermans.

A report on the First International Colloquium on Rheological Problems in Biology held in 1950 is included with abstracts and discussions. A listing of the literature referred to in the essays, an author index, and a subject index complete the volume.

Annual Report of the Executive Secretary-Treasurer of the American Society of Plant Physiologists for 1951–1952.—The report on the financial status of the A.S.P.P. has been presented by Dr. J. Fisher Stanfield. The favorable condition of the Society's affairs reflects the continuous active support given by its members. Some interesting developments are shown in the following figures. Membership: Active members July 1, 1951, 941; July 1, 1952, 1007, for a net gain of 66 members for the year. These members include: 29 Life Members, 16 Corresponding Members, 7 Institutional Members, 23 Canadian Members, 870 U. S. Members, and 67 Foreign Members. Subscriptions: Volume 26 in U. S., 363; Foreign, 522; Total for Volume 26, 885. Volume 27 in U. S., 375; Foreign 617; Total for Volume 27, 992, representing a net gain of 107 subscriptions during the year. The number of paid copies of PLANT PHYSIOLOGY mailed to regular members and subscribers for July 1952 totaled 1962.

These increases in membership and subscriptions exemplify the growth of the Society. It seems fitting to mention here that the increase of membership and subscriptions reflect, in large measure, the continued efforts of Dr. J. Fisher Stanfield in this direction. Members are asked to send to the Executive Secretary-Treasurer, at any time, the names and addresses of persons interested in and eligible for membership.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

Organized in 1924 for the advancement of Plant Physiology as a pure and applied field of botanical science

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Any one actively interested in Plant Physiology is eligible for membership in the Society. Candidates for membership must be recommended by two members, and approved by executive committee, or by the vote of the Society at some annual or special meeting.

The payment of the annual dues to the Society entitles the members to receive the official journal, Plant Physiology. They will receive in addition the regular bulletins and announcements sent out periodically by the secretary. Members are invited to take a personal interest in the growth and development of the Society.

Manuscripts for Plant Physiology should be addressed to Prof. Walter F. Loehwing, Department of Botany, State University of Iowa, Iowa City, Iowa. Drawings and photographs should not exceed 8.5 × 11 inches in size, should be numbered correctly on the back, and separate typewritten legends included with the text of the manuscript. Tables and cuts should be used sparingly, and the text presented in concise form. Instructions to authors for preparation of manuscripts are given in the January number, Volume 27, 1952, of Plant Physiology, 221–222.

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